BINDING SITES IN THE NUCLEAR ENVELOPE FOR CYTOPLASMIC GLUCOCORTICOID RECEPTOR COMPLEX

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CERTIFICATE OF SUPERVISOR

In terms of paragraph eight of 'General regulations for the degree of Ph.D.' I, as supervisor of the candidate, P.J. Smith, certify that I approve of the incorporation into this thesis of material that has already been published or submitted for publication.

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SUMMARY

Using tritiated triamcinolone acetonide to monitor purification, cytoplasmic triamcinolone acetonide-receptor complex has been purified 3 000 fold from rat liver cytosol. The isolated complex sedimented as a single radioactive peak on a 5 - 20% sucrose gradient. Nuclear envelopes isolated from purified rat liver nuclei were found to contain binding sites for the partially purified cytoplasmic triamcinolone acetonide-receptor complex. The binding constants showed two saturable high affinity binding sites and the envelope bound the complex with a specific activity ten times higher than the plasma membrane and more than three times higher than the two endoplasmic types of membrane. Saturable binding to chromatin was not observed in the concentration range tested. Free steroid hormone did not bind the envelope.

Binding sites for steroid hormones or steroid hormone-receptor complexes have been demonstrated both in chromatin and the nuclear protein matrix (Barrack and Coffey, 1980; Spelsberg, 1976). Because the nuclear envelope may be isolated with both these nuclear subfractions, the observed binding sites for steroid hormone-receptor complexes might be due to the presence of envelope components. The extent of association of nuclear envelope or nuclear envelope components with chromatin and the matrix was therefore investigated. Nuclear envelope fragments could be isolated from chromatin purified by centrifugation through 1,7 M sucrose. The binding of triamcinolone acetonide-receptor complex to these fragments was indistinguishable from the binding to purified nuclear envelope. A certain class of saturable chromatin binding sites for steroid hormone-receptor complexes may thus be due to the presence of envelope fragments. Extensive association of nuclear envelope polypeptides with the nuclear protein matrix was also observed. The matrix however, failed to bind triamcinolone acetonide-receptor complex.

The nuclear envelope comprises an inner and outer membrane with well defined pore complexes spanning both membranes. In order to identify the location of the binding sites for trimacinolone acetonide-receptor complex in the envelope, fractionation and reconstitution of

envelope proteins and lipids was attempted. Envelopes were solubilized in 2 - chloroethanol and protein and lipid components separated by chromatography on Sephadex LH 20. Envelope protein and lipid could be successfully reconstituted from chloroethanol by dialysis against aqueous buffer. Results showed that the receptor complex binds to the protein rather than lipid component of the envelope. This component was extractable by concentrations of the nonionic detergent Triton X-100 which do not extract the pore complex or lamina components of the envelope and is therefore probably a loosely bound membrane protein.

The presence of specific binding sites for triamcinolone acetonide-receptor complex on the nuclear envelope may be necessary for the transport of the complex into the nucleus. The possibility that the envelope mediates the glucocorticoid response in ways not linked to transport of the cytoplasmic receptor complex into the nucleus cannot be ruled out.

PART 1

INTRODUCTION

1.1 INTRODUCTION

"Although extensive data are now available about many aspects of glucocorticoid hormone action, the fundamental mechanism through which receptor-glucocorticoid complexes act remains unknown. Although it is widely propounded that these complexes interact with chromatin and regulate specific mRNA levels, it remains to be answered how such an interaction causes changes in gene expression. What regulatory factors lie between this interaction with the nucleus and the appearance of specific mRNA's? What explains the selectivity of glucocorticoid response?" (Johnson et al., 1979) At this point in time there has been no unequivocal identification of the nuclear acceptor for any steroid hormone receptor complex.

In the classic model of hormone action, peptide hormones were considered as intercellular signals which evoked, by interacting with the cell membrane, the accumulation of specific "second messengers" (e.g. cAMP) in effector cells. The model developed during the last decade for steroid hormones deviates from this in that the hormone itself, complexed to a cytosolic receptor is postulated to have the primary effect on the genome.

A central dogma has been developed in recent years in the biochemical literature to describe the general mechanism of action of steroid hormone action. The main features of this model, which in reality at this point in time is at best a working hypothesis, are outlined in Figure 1.1.

Firstly the steroid penetrates the cell membrane and binds to a specific receptor protein in the cytosol (Beato et al., 1970; Giannopolous, 1975). The presence of these receptors represents the first level of specifity characteristic of steroid responsive cells. Once bound to hormone, the receptor is then activated for transport to the nucleus. The nature of this

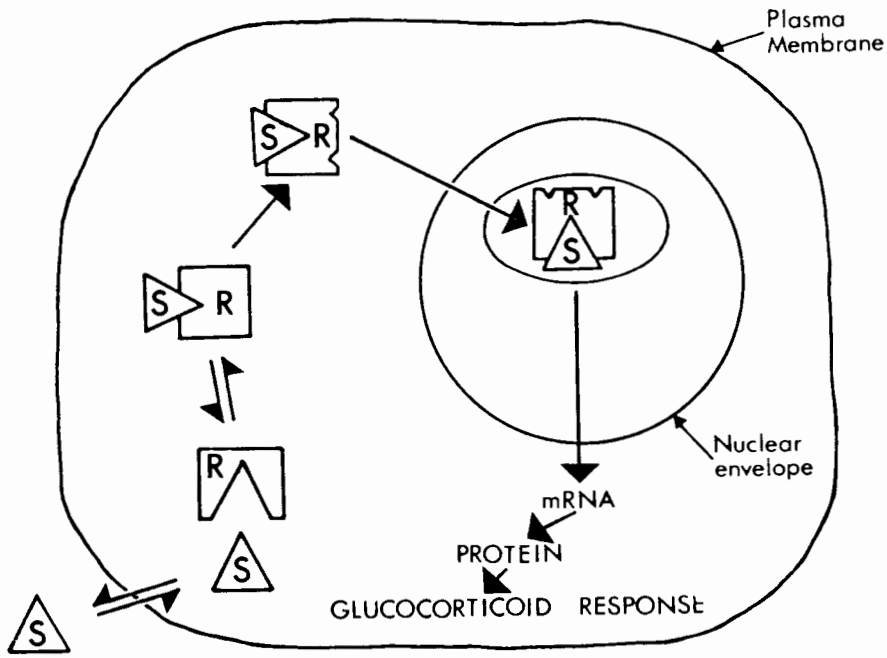

FIGURE 1.1 Schematic outline of proposed mechanism of steroid action

activation "in vivo" is unknown but may be brought about "in vitro" by a number of procedures including brief heating or by exposure to high salt. Once in the nucleus, the mechanism of action leading to stimulation of synthesis of specific mRNA's is unknown. This mechanism is presumed to involve interaction of the steroid-receptor with chromosomal proteins and/or DNA, leading to derepression of certain genes.

The time frame of the above events has been well described for estrogen in chick oviduct system by O'Mally and others (Thrall et al., 1978). One minute after injection, labelled hormone can be detected in target and nontarget cells. Within 1 - 2 minutes the steroid binds to its cytosolic receptor. Within 5 minutes, the steroid becomes predominantly located in the nucleus. Nuclear binding is followed by activation of RNA polymerase at 5 - 10 minutes, RNA synthesis occurs from 5 - 30 minutes, protein synthesis 15 - 30 minutes and an eventual increase in chromatin template activity at 1 - 2 hours post injection.

A number of authors (Johnson et al., 1979) have emphasized the analogy of regulation of gene expression by cyclic AMP in E.Coli with the proposed glucocorticoid action in eukaryotes. In E.Coli, cyclic AMP is produced

in response to glucose deprivation. It binds a specific "CAP" protein and this complex in turn binds DNA. The complex binds with ten times higher affinity to promoter regions of the DNA than to non-promoter regions although the precise reason for this is unclear. Among other effects,glucocorticoids also increase glucose production in eukaryotes due to induction of gluconeogenic enzymes. However, in the more complex eukaryotic genome it may equally be possible that gene expression by glucocorticoids is a catalytically mediated event rather than one involving direct stoichiometric interaction of the steroid or the steroid hormone-receptor complex with specific sites of the genome.

The possibility that catalytic mechanisms are operative at the nuclear level and may mediate glucocorticoid action seems likely in the light of the known role of protein modifications in eukaryotic regulation and hormone action (e.g. phosphorylation, cAMP action).

Both histones and nonhistones are extensively phosphorylated in eukaryotic chromatin on activation of transcription or replication (Allfrey et al., 1973). Phosphorylation of H_1 has been found to be greater in proliferating than quiescent cells (Balhorn et al., 1971) but the significance of this is not known. Nonhistones are also phosphorylated by a number of different protein kinases, some of which are stimulated by cAMP (Balhorn et al., 1971). Allfrey (1973) has observed that phosphorylation of rat liver nuclear proteins was increased within 30 minutes of a single cortisol injection. In the case of histone acetylation, Libby (1973) found that within minutes of administration of glucocorticoids to rats, there is an increase in acetylation of the core histones of the liver.

The glucocorticoid response has not as yet been shown to involve the induction of cAMP. Induction of a cyclic nucleotide at the nuclear level as a response to the steroid would presuppose the presence of a nucleotide cyclase in the nuclear envelope, analogous to the plasma membrane bound adenylate and guanylate cyclase. This enzyme however has not so far been identified in the nuclear envelope. Neither has the ability of cAMP to act directly in the eukaryotic nucleus been demonstrated, though nuclear cAMP binding proteins have been described (Kallos, 1977).

Although direct induction of cAMP by glucocorticoids had not been observed, glucocorticoids do influence the cAMP sensitive phosphorylation of a particular protein in amphibian and mammalian target tissues (Liu and Greengard, 1976). It has been reported that the progesterone receptor has ATP-binding and pyrophosphate exchange activities (Moudgil and Toft, 1976) and more recently ATP had been observed to play a part in the activation of cytosolic glucocorticoid receptors (John and Moudgil, 1979; Sando et al., 1979b).

Which signals, if any, mediate glucocorticoid receptor action, and to what extent they do, remains unsettled. So far virtually every component in the nucleus has been suggested as the acceptor which binds the cytosolic hormone-receptor complex in vitro. Examples are the nuclear envelope (Jackson and Chalkley, 1974a), the ribonucleoproteins (Liao et al.,1973), histones (Sluyser, 1969), basic nonhistones (Mainwaring et al., 1976), acidic nonhistones (King et al., 1969;Defer et al., 1974; Spelsberg et al., 1977) and DNA (Baxter et al., 1972; Andre and Rochefort, 1975). There have also been reports of specific binding of free steroid hormone to nuclear material (Liu and Greengard, 1976). Consequently the chemical identity of the nuclear acceptors has yet to be determined. Lack of clear evidence of the location of the binding site of the steroid hormone-receptor complex in the nucleus has complicated attempts to decide whether changes in gene expression caused by glucocorticoids are catalytically mediated or due to direct derepression by the hormone receptor complex.

Autoradiographic analysis of labelled steroids in target cell nuclei after injection into whole animals or incubation with isolated cells revealed about 25% of the activity closely associated with the nuclear envelope and 75% with the nucleoplasm (Jenson et al., 1969). Of the latter there is no apparent localization of radioactivity around the nucleolus or other intranuclear regions. In vitro experiments indicate over 90% of labelled steroid associated with isolated chromatin.

Because isolated chromatin shows a high degree of binding of steroid receptor complexes, this nuclear fraction has been most extensively investigated in the search for the nuclear acceptor for these complexes (for reviews, see Thrall et al., 1978, O'Malley et al., 1977). Studies with a number of different steroids appear to implicate both DNA and the

nonhistone proteins or a DNA-non-histone complex as potential acceptor sites. Similar results have been obtained with corticosteroids (Ichii and Murakami, 1978; Cidlowski and Munck, 1978; Climent et al., 1977; Rousseau et al., 1975; Bugany and Beato, 1977; Defer et al., 1976; Simons et al., 1976; Milgrom et al., 1976; Giannopolous, 1977; Hamana and Iwai., 1978).

The involvement of a DNA-nonhistone complex as an acceptor site is an attractive finding for those who favour the analogy of the mechanism of action of the steroid-receptor complex in eukaryotes with that of the cAMP-CAP complex in prokaryotes. The simplest interpretation of all the results is that DNA is involved in acceptor activity and nonhistones and histones act to influence or direct the binding. Two factors complicate this model. In the first place, steroid hormone-receptors all bind DNA non-specifically (Rousseau et al., 1975; Milgrom et al., 1976). Secondly, the extent of contamination of chromatin by nonchromatin components is unknown and presently not measurable. Thrall (1978) has sounded a note of caution concerning the non-chromatin origin of many components of isolated chromatin. One of the problems which arises during subfraction of the nucleus is whether isolated subfractions may be reliably equated with their morphological namesake observed "in situ". Chromatin, which has a well defined "in situ" location, is the most obvious example. In practice, isolated chromatin may contain every component of the nucleus. Consequently the localization of any acceptor sites on isolated chromatin, in reality may represent a non-chromatin entity. Nonhistone proteins implicated in hormone-receptor binding may thus be of non-chromatin origin.

The interaction of steroid hormones or steroid hormone-receptors with non-chromatin components of the nucleus has received scant attention. This is particularly true for the nuclear envelope which is a persistent contaminant of isolated chromatin (Wong et al., 1973, Eisen and Glinsman, 1976; Climent et al. 1976). A few reports have noted the binding of steroid hormones to the nuclear envelope (Jackson and Chalkley, 1974; Lefebvre and Nevsad, 1980) but most authors assume that the cytoplasmic steroid hormone-receptor complexes enter the nucleus by diffusion through the nuclear pores. The interplay of information-bearing molecules between cytoplasm and nucleus is poorly understood and although the molecular changes involved in the nuclear transportation of steroid hormone-receptor complexes have been investigated extensively, only limited information is available on their

intra-cellular movement. ^{3}H estradiol has been observed by autoradiography to be located on the nuclear envelope (Disorbo et al., 1980) as has ^{3}H testosterone (Frederik et al., 1977). Nenci et al (1980) using immuno-fluorescence techniques, found that estradiol-receptor complex binds chiefly to the nuclear envelope and is localized mainly around the nuclear pore complexes.

If chromatin is indeed the final destination of the cytoplasmic steroid hormone-receptor complex, then the complex must of necessity negotiate the nuclear envelope. It is unlikely that it would do this via simple diffusion through the pores in view of the rapidity of nuclear translocation after the steroid has bound to its cytosolic-receptor (Thrall et al., 1978). This would therefore indicate a specific interaction of steroid hormone-receptor with the envelope.

Apart from the involvement of the nuclear envelope in transport of steroid hormone-receptors from the cytoplasm into the nucleus, it is quite possible that the envelope itself may be the target for specific cytoplasmic hormonal signals which through catalytic mechanisms lead to increased nuclear activity, more in line with the mode of action of peptide hormones. The nuclear envelope may thus well be considered as the first relay station in such cytoplasmic-nuclear interaction. Whether interaction of steroid hormone-receptor complexes with the nuclear envelope is simply a step preceding transport into the nucleus or is an infomation-bearing step, or both, is an open question.

Some of the problems currently encountered in locating the nuclear sub-fraction which specifically binds cytoplasmic steroid hormone-receptor complex are considered below.

1.2 THE GLUCOCORTICOID-RECEPTOR COMPLEX

1.2.1 Isolation and Characterization

"In vitro" experiments with steroids in general are hampered by methodological constrains, one of the chief being the difficulties involved in obtaining a homogeneous preparation of cytosolic steroid hormone-receptor complex.

Rat liver cytosol contains at least four separate cytosolic glucocorticoid binding proteins, Binders I - IV (Koblinsky et al., 1972; Beato and Feigelson, 1972; Litwack et al., 1973), only one of which translocates to the nucleus on activation (Litwack et al., 1978).

A number of these cytosolic glucocorticoid-binding proteins have been identified. Beato and Feigelson (Koblinsky et al., 1972; Beato and Feigelson, 1972) found three thermolabile binders in rat liver cytosol which they called A, B and G binders. A and B had a molecular weight of 51 000 and 64 000 daltons respectively whereas the G protein had a weight of 200 000 daltons at low ionic strength and about 66 000 daltons at a 0,3M NaCl concentration. In a separate study, Litwack et al (1973) identified four binding proteins which they called binders I - IV. The B protein and Binder IV have been identified as transcortin and Binder I as ligandin. Binder III appears to be a small breakdown product of about 5 000 daltons. Only Binder II and the G protein translocate to the nucleus and would appear to be the same protein. Litwack's group has recently identified another binder IB (Litwack and Rosenfield, 1975) which appears to bind unmetabolized glucocorticoids (Litwack et al.,1978).

Binder II (G protein) is accepted as the nuclear translocating protein which mediates the glucocorticoid effect. It is also the only cytosolic binding protein which binds 9α-fluoro glucocorticoid derivatives (Koblinsky et al., 1972). 9α-fluoro substitution in glucocorticoids has been shown to increase specific binding to the receptor (Munck and Brinck-Johnson, 1968). Dexamethasone and triamcinolone acetonide, both 9α-fluoro glucocorticoids analogues, are frequently used in the place of natural glucocorticoids. They both form a more stable complex with the receptor (Wrange,1976).

There have been queries as to whether the synthetic steroids bind the same proteins as the natural glucocorticoids (Agarwal, 1976;Agarwal, 1977; Feldman et al., 1978). Competition experiments indicate that corticosterone and dexamethasone interact with the same protein (Wrange, 1976), but Agarwal (Agarwal, 1976, Agarwal, 1977) finds that dexamethasone and corticosterone complexes elute under different conditions from a DEAE 52 column. Although this could indicate different receptors, it

might simply indicate modifications of the same receptor due to binding different steroid hormones.

The basis for the partial purification of cytosolic glucocorticoid-receptor complex currently rests on the ability of the activated form of the complex to bind to polyanions such as DNA or phosphocellulose (Climent et al., 1976; Eisen and Glinsman, 1976; Eisen and Glinsman, 1978; Govindan and Sekeris, 1978; Atger and Milgrom, 1976; Westphal and Beato, 1980). The nonactivated form does not. Prior to an activation step the charged cytosol is passed over a DNA cellulose or phosphocellulose column to remove other steroid binding proteins which bind to polyanions. The glucocorticoid receptor complex is then activated and the cytosol passed over the polyanion column to which it now binds. The activated steroid hormone-receptor complex is then eluted from the column with high salt (0,35M NaCl) or more recently with 10mM pyridoxyl phosphate (Disorbo et al., 1980; Dolan et al., 1980). The use of pyridoxyl phosphate as eluant leads to a modification of a lysyl residue (or residues)through formation of a Schiff base. These residues thus appear to be essential for the binding of the receptor to DNA or phosphocellulose (Disorbo et al., 1980). This modification also disrupts binding of steroid hormone-receptors to acceptor sites in nuclei (Müller et al., 1980; Dolan et al., 1980). Further steps such as ammonium sulphate precipitation and DEAE cellulose chromatography have also been included (Eisen and Glinsman, 1976; Govindan and Sekeris, 1978; Westphal and Beato, 1980). Initial chromatographic steps on phosphocellulose must be performed rapidly, as prolonged exposure to phosphocellulose has been shown to activate the hormone-receptor (Atger and Milgrom, 1976).

The above methodology leads to the isolation of the activated form of the steroid hormone-receptor complex. The nonactivated form is less easily isolated, although affinity chromatography utilizing immobilized steroid has been employed (Wong et al., 1973; Govindan and Sekeris, 1976; Govindan and Sekeris, 1978). Elution of the receptor from the affinity column is difficult due to the high affinity of the steroid for the receptor. This problem has been overcome by binding the steroid to the matrix through an "arm" containing a disulphide bond. Govindan and Sekeris (Govindan

1976; Govindan and Sekeris, 1978; Govindan and Manz, 1980) linked 11-deoxycortisone to Sepharose 4B via a disulphide bond and eluted the hormone receptor complex with β-mercaptoethanol. This method isolates all cytosolic steroid binding proteins and not just the one which translocates to the nucleus. More recently Govindan (1980) has isolated rat liver nuclear glucocorticoid receptor complexes using antibody bound to Sepharose 4B. The antibody was generated using affinity cytosolic glucocorticoid receptor complex. Eisen (1980) has performed similar experiments.

Agreement on the size or subunit nature of the activated receptor complex has yet to be reached. Beato et al (Westphal and Beato, 1980) obtain a single receptor of 40 000 daltons whereas other workers find as many as three receptors (Govindan and Sekeris, 1978; Govindan, 1980; Govindan and Manz, 1980; Tsawdaroglou et al., 1981) of molecular weights 45 000, 72 000 and 90 000 daltons. Eisen et al (1981), using dexamethasone 21-mesylate to affinity label the rat liver glucocorticoid receptor obtained three labelled proteins of 90 000, 67 000 and 52 000 daltons. The largest protein was identified as the receptor and the other two as albumin and transcortin respectively. Westphal et al (1981) have photoaffinity labelled a 40 000 dalton triamcinolone acetonide receptor purified from rat liver cytosol. Whether the smaller receptors are subunits or proteolytic breakdown products of the larger complexes has not been established although antibodies elicited to the various receptors have been shown to cross-react with a number of the others (Tsawdaroglou et al, 1981).

The identity of the activated cytosolic glucocorticoid-receptor complex and the nuclear bound complex is disputed. Govindan (1980) has found the two complexes to be identical. However, glucocorticoid-receptor complexes isolated from rat liver nuclei by Wrange and co-workers (Carlstedt-Duke et al., 1977, Wrange and Gustafsson, 1978) do not have the same value or Stoke's radius as cytosolic receptors. Indications are that the smaller receptor (Stokes radius of 3,6nm as opposed to 6,1nm) extracted from nuclei may be a breakdown product of the larger cytosolic receptor. The evidence for this comes chiefly from observations that a smaller 3,6nm receptor could be generated from the larger 6,1nm subunit in low ionic strength. Both complexes are taken up by nuclei. Trypsin treatment of the 6,1nm receptor (Wrange and Gustaffson, 1978) generated smaller steroid binding fragments of 3,6nm and 1,9nm the latter of which does not translocate to the nucleus. However, the possibility that the nuclear receptor is an altogether different protein cannot be ruled out.

Glucocorticoids, like other steroid hormones,exert their effect at very low concentrations of between 10^{-7} - 10^{-8}M (Hamana and Iwai, 1978). Equally the concentration of cytosolic receptors is very low and detection of the hormone-receptor complex through various purification steps can only be easily monitored using a radioactive steroid. In most cases the protein concentration is too low for detection by conventional methods and detection by gel electrophoresis is hampered by the fact that the steroid dissociates from the receptor under most electrophoretic conditions. A reported purification of 60 000 fold has yielded an almost homogenous preparation of the complex. Yields vary immensely from 2,7% (Westphal and Beato, 1980) to 85% (Eisen and Glinsman, 1978).

The main difficulties faced when attempting to purify the glucocorticoid receptor complex can be grouped under the following headings:

1) limited availability of a tissue abundant in free receptor
2) the presence of proteins other than the receptor which bind glucocorticoids
3) the high degree of purification necessary to obtain homogeneous preparations and
4) the apparent instability of all forms of receptor.

1.2.2. Activation

So far, activation is largely an operational definition to describe the transformation of steroid hormone receptor complexes that must occur before association with nuclear acceptor sites is observed.

Rousseau et al (1973) observed that whereas at 37°C, the dexamethasone-induced transfer of cytoplasmic receptors to nuclei of hepatoma (HTC) cells is complete within 30 minutes, at 0°C nuclear transfer proceeds very slowly. Increasing the temperature to 37°C causes immediate association of the steroid-receptor complexes with the nucleus. This temperature-dependent nuclear binding of glucocorticoid receptors has been found in many systems including thymocytes (Munck et al., 1972), foetal lung (Giannopolous, 1975), pituitary (Wanatabe et al., 1974), and mouse fibroblast cells (Ishii et al., 1975). From this finding has arisen the suggestion that binding of a steroid hormone to the cytoplasmic receptor is itself insufficient for association of the complex with the nucleus. This temperature-sensitive step belongs to the cytosol since

binding to isolated nuclei takes place at 0°C if the cytosol, but not the nuclei is first incubated at higher temperature (Baxter et al., 1972).

Thermal energy is not the only factor involved in activation of steroid hormone-receptor complexes. Increasing the ionic strength can mimic this effect (John and Moudgil, 1979; Munck and Foley, 1976; Bailly et al., 1978; Bailly et al., 1980). The receptor is also activated by dilution of the cytosol or removal of a proposed low molecular weight inhibitor by gel filtration (Goidl et al., 1977). Prolonged exposure to DNA or phosphocellulose leads to progressive activation of complexes (Le Fevre et al., 1979). Recently ATP has also been implicated in activation (John and Moudgil, 1979; Moudgil and John, 1980a; Moudgil and John, 1980b). Incubation of cytosol in low salt at 4°C with 5 - 10mM ATP produces a hormone-receptor capable of binding to nuclei. In mouse fibroblast cells inactivated hormone-receptors have been reactivated with 10mM ATP (Sando et al., 1979b). Other nucleotide triphosphates or cyclic nucleotides have no effect. However, Andreasen (1981) found inhibition of activation by 1,6 millimolar concentrations of ADP and ATP in 0,4M KCl at 0°C. The inhibition was counteracted by millimolar concentrations of theophylline and $MgCl_2$. The actual mechanism of activation has not yet been elucidated.

Active and inactive glucocorticoid-receptors cannot be distinguished by gradient sedimentation analysis (Giannopoules et al, 1973; Kalimi et al., 1975), although uptake by nuclei appears to involve the conversion of a receptor with a Stokes radius of 6,1nm to one with a Stokes radius of 3,6nm (Carlstedt-Duke et al., 1977). This conversion can be effected by proteolytic enzymes (Wrange and Gustaffson, 1978). Kinetic data shows the reaction to be first order, yielding a monomolecular product which is in equilibrium with the nonactivated form (Atger and Milgrom , 1976). Bailly et al (1978) found that heat activation increases the rate of the reaction but does not affect the equilibrium between activated and non-activated complexes, whereas increasing ionic strength, pH, or removal of a low molecular weight inhibitor, increases the concentration of activated complexes at equilibrium. It appears that the various activation procedures act independently of each other and additively.

The role of phosphorylation and dephosphorylation in both the steroid hormone binding ability and activation of the cytosolic receptor has been considered by a number of workers (Sando et al., 1979a; Maki et al., 1980). Pratt's group have proposed that reduction and phosphorylation of the receptor are necessary for steroid binding. They and others (Maki et al., 1980;Noma et al., 1980) found that molybdate, glucose-1-phosphate and fluoride, all phosphatase inhibitors, stabilize the steroid hormone-receptor complex. 2mM dithiothreitol also had a large stabilizing effect. The presence of ATP also enhances steroid binding to the receptor (Sando et al., 1979a). Barnett et al., (1980) have found that certain phosphatase inhibitors inhibit activation of triamcinalone acetonide-receptor complexes in rat liver, whereas calf intestinal alkaline phosphatase stimulates the rate of activation. They propose that while phosphorylation may be necessary for steroid binding, a dephosphorylation reaction initiates activation. There is no evidence yet whether either phosphorylation or dephosphorylation occurs on the steroid hormone-receptor itself or on some other regulatory components.

The role of pyridoxyl phosphate in activation and DNA binding has been investigated by a number of workers (Müller et al, 1980; Dolan et al, 1980). Westphal and Beato (1981) showed that millimolar concentrations of pyridoxyl phosphate were able to inhibit not only receptor binding to DNA but also that of a heterogeneous population of hepatic proteins and the lac repressor of E. Coli. They conclude that the action of pyridoxyl phosphate is thus a general rather than a specific one. O'Brien and Cidlowski (1980) reported pyridoxyl phosphate mediated disaggregation of glucocorticoid-receptors from HeLa S_3 cells. More recently (Sekula et al, 1982) pyridoxyl phosphate has been shown to exert both a stimulatory effect on activation as well as an inhibitory effect on binding of activated complexes to DNA. The physiological significance of these effects has yet to be demonstrated. An endogenous cytosolic micromolecule has been shown to inhibit activation (Sekula et al, 1981), but still awaits characterization.

10 mM sodium molybdate has also been shown to inhibit activation (Jones and Bell, 1982; Dahmer et al, 1981; McBlain et al, 1981). Tungstate, another group 6A metal oxyanion, prevents nuclear uptake of heat activated receptor complex (Murakami et al., 1982).The mechanism of action awaits elucidation. Leach et al (1982) have reported a heat stable cytosolic fraction which, like molybdate, inhibits activation of receptor to the DNA binding state.

Whereas the activation process can be demonstrated "in vitro", questions have been raised as to whether this is an "in vivo" phenomenon. Munck and Foley (1980) showed that when glucocorticoids initially enter rat thymus cells incubated at 37°C, nonactivated complexes are formed within 15 seconds and then rapidly replaced by activated complexes. The activated and non-activated complexes were separated on a DEAE cellulose column by elution with different concentrations of salt. Markovic and Litwack (1980) obtained similar results in rat liver cytosol. The earliest measurements taken 5 minutes after hormone injection produced about 40% activated and 50% unactivated complexes. After 60 minutes only 10% of complexes remained unactivated. These observations, for the first time using the whole animal, indicate that the activation of glucocorticoid-receptors is a physiologically significant process.

The current understanding of the process involved in activation of the steroid hormone-receptor complex is by no means complete. Studies on the complex are complicated by the labile nature of partially purified preparations. Activated glucocorticoid receptors are unstable at 37°C and tend to aggregate with unknown cytosol proteins (Bulanyi and Oliver, 1976). They do not withstand lyophilization but are stabilized by bovine serum (1mg/ml) (Climent et al., 1976) and may be stored for months in liquid nitrogen (-170°C).

1.2.3. Nuclear Translocation

Activation and uptake by the nucleus distinguish the glucocorticoid receptor from other glucocorticoid binding proteins in the cytoplasm (Beato et al., 1970; Giannopolous, 1975). The receptor does not translocate to the nucleus unless charged with steroid (Giannopolous, 1975). This finding has been recently challenged by Papamichael et al., (1981) who found that in PHA stimulated human lymphocytes, translocation of receptor from cytoplasm into the nucleus took place in the absence of glucocorticoids.

Autoradiographic studies show "in vivo" accumulation of radiolabelled steroid in the nucleus and cell fractionation and immunocytochemical experiments indicate an accumulation of steroid in the nucleus of target cells (Papamichael et al., 1980; Rousseau et al., 1973). In hepatoma tissue culture (HTC) cells exposed to dexamethasone at 37°, nuclear binding of steroid reaches a maximum with 30 minutes and then levels off (Rousseau et al., 1973). Concomitant with nuclear binding there occurs a depletion of receptors from the cytosol equivalent to the quantity of steroid taken up by the nucleus. Removal of hormone from the incubation medium, results in a disappearance of hormone from the nucleus and a re-appearance of cytosolic receptors.

Regarding the number of receptors per cell and the percentage which translocate to the nucleus, results have varied. Beato et al.,(1974) find 60 000 binding sites per rat liver cell and 15 000 per nucleus. However in HTC cells roughly the same number of binding sites was observed for both whole cells and nuclei (Rousseau et al., 1973). There is also disagreement as to whether the distribution of steroid between cytosol and nucleus is concentration dependent or not (Beato et al., 1974; Pfahl et al., 1978). Most authors find that nuclear binding of the steroid hormone-receptor is non saturable although some authors do find saturation, particularly at physiological ionic strength (Higgins et al., 1979). The "in vivo" binding constant is not known but is estimated at being above 3nM, as this is the concentration of free activated complexes in HTC cells.

The kinetics of nuclear binding suggest that the association is of high affinity and involves a limited number of a single class of nuclear acceptor sites (Baxter and Tomkins 1970). However Atger and Milgrom (1978) reach the opposite conclusion. They have achieved saturation of rat liver nuclei using a 940 fold purified steroid hormone-receptor complex but find that the number of acceptor sites greatly exceeds the cellular concentration and suggest that this saturation does not take place "in vivo". At high steroid concentration, nuclei have also been found to

accumulate transcortin-bound as well as free steroid (Seleznev et al., 1979). This can account for up to 30% of nuclear bound steroid hormone. Bulanyi and Oliver (1976) sounded a word of caution in respect of the interpretation of nuclear binding in experimenting where the integrity of the cell has been destroyed. They showed that a substantial proportion (67%) of radioactivity associated with a low speed nuclear pellet is in fact due to aggregation and coprecipitation of the hormone-receptor complex and leads to an overestimation of the number of true nuclear acceptor sites.

One of the accepted criteria indicative of specific binding of the steroid hormone-receptor complex to the nucleus is the extractablility of the complex with 0,35 M NaCl or KCl. Some authors have found that only a proportion of the complex is extracted with high salt and this has led to the proposal that there may possibly be two classes of acceptor site - a low and a high affinity one (McPartland et al., 1977; Pfahl et al., 1978). Pfahl et al., (1978) suggest that these results are comparable to binding of the cAMP-CAP complex in E.Coli, where a large number of low affinity and small number of high affinity sites are observed.

The question of the tissue specificity of nuclear binding of glucocorticoids is difficult to approach as nearly all tissues possess receptors for glucocorticoids (Ballard et al., 1974) and it is hardly surprising that their nuclei are accceptor positive. The immature rat uterus is one tissue devoid of glucocorticoid receptors (Ballard et al., 1974; Higgins et al., 1973), yet uterine nuclei are also acceptor positive (Higgins et al., 1973). The sex hormone-receptors appear to have a degree of tissue specificity (Webster et al., 1976). However this problem has not yet been satisfactorily resolved and still remains an open question.

The necessity of activation of the hormone-receptor complex as a pre-requisite for nuclear binding has already been mentioned, and the variety of factors affecting the activation process will naturally also affect nuclear binding. Heterogeneity of steroid hormone-receptor complexes in cell free experiments infinitely complicates otherwise simple equilibrium reactions. There is the inherent difficulty in distinguishing between activated and non-activated steroid hormone-receptor complexes in experiments using whole cytosol as the complex is detectable due only to the incoporation of a radioactive label in the steroid. The cytosol also

contains heat stable inhibitors of activation (Milgrom and Atger, 1975). Partial purification of the activated steroid hormone-receptor eliminates the problem of heterogeneity but the problem of nonspecific aggregation and possible artefactual entrapment of the complex due to clumping nuclei or binding to DNA released from burst nuclei remains (Traish et al., 1977; Müller et al., 1977).

Hence problems abound in the detection of the number of biologically active acceptor sites in the nucleus. The prospect of pure and well characterized steroid hormone-receptor complexes becoming a common occurrence (Climent et al., 1976; Govindan and Manz, 1980) should help to eliminate many of the problems mentioned.

1.3. NUCLEAR LOCALIZATION OF THE GLUCOCORTICOID RECEPTOR COMPLEX

1.3.1. Chromatin and DNA

The localization and nature of the nuclear acceptor for steroid hormone-receptor complexes in general and glucocorticoid-receptors in particular has been the subject of extensive research over the last ten years. However, this has thrown little light on the details of the biochemical events occurring after nuclear binding of the activated steroid hormone-receptor complex. Virtually every subfraction of the nucleus has been proposed to contain the acceptor site for cytosolic steroid hormone-receptor complexes (see 1.1). The main sites favoured as targets for the steroid hormone-receptor are chromatin, DNA and/or the so-called nonhistone fraction (Ichii and Murakami, 1978; Rousseau et al., 1975; Simons et al., 1976; Hamana and Iwai, 1978).

Receptors for all steroid hormones bind to chromatin isolated from their respective target tissues (Higgins et al., 1979). Chromosomal proteins have long been candidates for the role of nuclear acceptor. Spelsberg and co-workers working on the progesterone-receptor complex, found that the acceptor appeared to be part of a nonhistone fraction they named AP_3. However this fraction only bound their receptor complex when it was complexed with DNA. Further work in this area (Spelsberg et al., 1976) indicated two proteins of molecular weight 12 000 - 17 000 daltons, both acidic, as acceptors in association with DNA. Such work has not been performed for the glucocorticoid receptor and Spelsberg's group has not made any recent advances in elucidation the precise nature of their

proposed acceptor complex. Climent et al., (1977) found that their partially purified triamcinolone acetonide-receptor complex from rat liver cytosol bound to nucleosomes, but that it could not distinguish rat and chicken nucleosomes. DNA itself appears to be important for nuclear or chromatin binding of glucocorticoid receptors.

Purified DNA binds the activated steroid hormone-receptor complex non-specifically and has been used coupled to cellulose in the isolation of the activated complex (Cidlowski and Munck, 1978). Glucocorticoid-receptor complexes failed to distinguish between DNA isolated from a variety of eukaryotes, prokaryotes and bacteriophages but did not bind to RNA (Rousseau et al., 1975; Simons et al., 1976; Milgrom et al., 1976). However RNA has been shown to inhibit steroid hormone-receptor binding to DNA-cellulose whereas the presence of RNAse led to increased binding (Chong and Lippman, 1982). Neither free receptors nor transcortin nor free steroid bound to DNA. Evidence for possible involvement of DNA in acceptor activity, comes from experiments using DNase I to partially digest DNA in intact nuclei. In HTC cell nuclei (Higgins et al., 1973) less than 10% acceptor capacity remained after one third of the DNA had been solubilized.

However, there are a number of differences in the binding of steroid hormone-receptors to DNA and nuclei or chromatin. Firstly binding to DNA is strongly inhibited at elevated ionic strengths. Rousseau et al.(1975) found 90% inhibition of binding to DNA at 100mM NaCl and total inhibition at 150mM NaCl. Similar results have been obtained by others (Bugany and Beato, 1977; Milgrom et al., 1976). Cidlowski and Munck however found that the presence of 0,15M NaCl decreased binding to DNA by only 17%. Secondly, the acceptor capacity of DNA is greater than that of nuclei but the binding affinity of the former is lower (Rousseau et al., 1975; Simons et al., 1976; Milgrom et al., 1976). Hamana and Iwai (1978) proposed that their 4S cytoplasmic steroid hormone-receptor combined with non-histones to form a salt extractable 6 - 7S complex which then bound to DNA. Bugany and Beato(1977) raised a note of caution when whole cytosol is used as a steroid hormone-receptor source as it contains DNases and could lead to a faulty assessment of DNA or chromatin binding. Climent et al (1977) noted that after digestion to core, the binding of steroid hormone-receptor to nucleosomes decreased markedly indicating that the complex probably binds spacer DNA.

Giannopolous (1977) found 2 forms of nuclear binding: 0,3M KCl soluble and insoluble. He also found that Triton X-100 released 20% of nuclear bound glucocorticoid radioactivity. 1% Triton X 100 has been found to remove as much as 60% of nuclear bound receptor complex. As the main effect of Triton X-100 is removal of virtually all membrane phospholipid (Jackson, 1976), these results indicate possible involvement of the nuclear envelope in nuclear binding.

Thrall and Spelsberg (1981) from studies on the interaction of chick oviduct progesterone receptor complex with DNA, concluded that minimal binding of the complex to pure DNA occurs under physiological conditions. An increase in binding could be obtained by lowering the pH or ionic strength. A decrease in pH from 7,4 to 6,4 resulted in an eight-fold increase in binding while a three-fold increase was obtained when the KCl concentration was decreased from 0,15 to 0,05 M. Hughes et al. (1981) were unable to find DNA-sequence specificity for the binding of chick oviduct progesterone-receptor complex to any region of the ovalbumin gene. However, in a recent study (Mulvihill et al., 1982) several cloned DNA fragments from chicken egg white protein genes were shown to compete with calf thymus DNA for the progesterone receptor. The recognition site was identified as a region 250 - 300 base pairs 5' - upstream from the transcription initiation site although fragments from other regions also competed for the receptor.

Recent work has also implicated DNA in the recognition of glucocorticoid-receptor complexes. Payvar et al.,(1981) found that glucocorticoid-receptor complexes bound specific sequences of mouse mammary tumour virus (MMTV) DNA which is strongly stimulated by glucocorticoids when introduced into the genome of a receptor containing cell. They found binding in areas both upstream and downstream from the transcription initiation site. Also using cloned MMTV DNA, Govindan et al.,(1982) were able to show binding to long terminal repeat sections of the DNA. There is some discrepancy between their results and those of Payvar et al.,(1981), but they suggest that this may be due to a difference in purity of the glucocorticoid-receptor complex used by each group. Both groups conducted their binding experiments under conditions well below physiological ionic strength.

Clearly, DNA has come back into contention as specific binding site for steroid hormone-receptor complexes in the nucleus, although the physiological significance of this binding has yet to be shown. Whether the derepression of a particular gene following interaction of a steroid hormone-receptor complex with chromatin acceptor sites, is the direct result of that stoichiometric event or the consequence of secondary catalytic events triggered by the receptor-acceptor interaction remains yet to be established. (See also Chapter 6.)

1.3.1.2 The Problem of Chromatin Contamination

The problems attending the precise localization of the acceptor site in the nucleus have been mentioned. In the first place DNA is able to bind the activated cytoplasmic receptor complex nonspecifically and hence any nuclear subfraction containing DNA is a candidate for nonspecific binding. Secondly, it is extremely difficult to obtain certain nuclear subfractions free from contamination by other fractions. This is particularly true for the nuclear subfraction called chromatin. Currently any treatment of the nucleus which disrupts its ultrastructure and releases readily soluble components, produces an insoluble fraction which may be called chromatin.

The nonhistone protein fraction of chromatin is even less well defined due partly to the diversity of starting materials used for its preparation i.e. crude chromatin (Paul and Gilmour, 1968; Shaw and Huang, 1970), chromatin purified through sucrose gradients (Bonner et al., 1968) or washed nuclei (Teng et al., 1971). Despite this there is no shortage of claims made for the role(s) of the nonhistone proteins in the control of eukaryotic gene expression (Bonner et al., 1973). Gilmour et al (1975) and Gilmour and Paul (1975) have demonstrated that acidic chromosomal proteins can stimulate the synthesis of specific mRNA transcripts from reconstituted chromatin. Nevertheless the intranuclear origin of the fraction of acidic protein responsible for this control remains unknown. It is possible that a large fraction of the nonhistones is not derived from chromatin at all.

One common source of possible chromatin contamination is the nuclear envelope. As DNA is attached at numerous positions to the inner nuclear membrane (Quick, 1980) this is not unexpected. However the nuclear envelope has been largely overlooked as a major chromatin contaminant for two main reasons: 1) The inclusion of a sedimentation step through 1,7M sucrose is presumed to remove all membrane contaminants (Bonner et al., 1973). 2) Treatment of either whole nuclei or chromatin with a nonionic detergent such as Triton X-100 is presumed, mainly on electron microscopic evidence, to remove the nuclear envelope (Blobel and Potter, 1966). Tata et al. (1972) have found substantial amounts of phospholipid in rat liver chromatin after centrifugation through 1,7M sucrose. The distribution of the phospholipids resembled that found in microsomal membranes. Further treatment of the chromatin with 1% Triton X-100 removed most of the phospholipid. However, although Triton X-100 destroys the bileaflet nuclear envelope structure, a residual single layer of collapsed membrane remains which is easily observed on electron micrographs.

It is widely accepted that when nuclei are treated with nonionic detergents the outer nuclear membrane is selectively removed (Blobel and Potter, 1966). Careful biochemical analysis of Triton extracts of nuclei, indicates that the detergent removes the bulk of membrane phospholipid but leaves a substantial insoluble protein residue (Jackson, 1976; Richardson and Agutter, 1980). Chief evidence for the selective removal of the outer nuclear membrane by Triton X-100 has been electron microscopic. After treatment with Triton X-100 the nuclear envelope bileaflet structure is replaced by a single layer in which the nuclear pore complexes are readily visible. This was assumed to be the inner nuclear membrane (Blobel and Potter, 1966). However, as this treatment removes over 80% of membrane phospholipid, but very little protein (Frederiks et al., 1978), the observed layer constituted rather a collapsed bileaflet structure than an intact inner membrane. There is considerable evidence in the literature that the Triton treatment preferentially removes phospholipid. Tata et al., (1972) demonstrated that even sheared chromatin still contains substantial amounts of phospholipid. Jackson (1976) has shown that nonionic detergent extraction procedures commonly employed in

preparation of acidic chromosomal proteins cannot be relied on to remove nuclear envelope polypeptides. Treatment of nuclei with 1% Triton X-100 removes about 83% of lipid but fails to remove a significant proportion of membrane protein. The bulk of protein extracted by this Triton treatment consists of histone material, indicating that rather than remove the outer nuclear membrane selectively, the detergent is able to penetrate the nucleus and solubilize histones. SDS gels of the nonhistone protein fraction and of the nuclear envelope appear virtually identical (Jackson, 1976).

Agutter and Richardson (1980) find that treatment of the nuclear envelope with 1% Triton X-100 removes 95% of phospholipid but only small amounts of protein. SDS gel electrophoresis of the Triton insoluble fraction produces a polypeptide pattern identical to that of the intact nuclear envelope indicating that the Triton is not selectively solubilizing a few polypeptides. A number of authors (Frederiks et al., 1978; Tata et al., 1972; Manzoli et al., 1978) make reference to the tightly bound chromatin lipid which survives rigorous Triton treatment and which they speculate to be of non membrane origin. This lipid, however, comprises less than 1% of total nuclear lipid. The bulk of non-solubilized chromatin lipid observed by Frederiks (1978) appears to be of membrane origin as the lipid distribution is similar to that of the nuclear envelope.

Persistence of nuclear envelope polypeptides in chromatin preparations from Triton-washed nuclei begs the question of the fate of membrane bound ribosomes. Dabeva et al.,(1977) investigating the removal of the nuclear envelope and its adhering ribosomes by Triton X-100 found that although neither the outer nuclear membrane nor ribosomes are visible electron microscopically, most of the ribosomal RNA has not been removed by Triton. Radioactively labelled RNA which survived detergent washes of nuclei was mainly 28S and 18S ribosomal RNA of cytoplasmic origin. Hence the RNA content of chromatin cannot be assumed to be contributed solely by HnRNA of nucleolar origin.

The conclusions reached by a number of authors that chromatin contains glycoproteins of nonmembranous origin have been based on the

supposition that nuclei and chromatin purified by the two criteria mentioned above are free of membrane contamination (Stein et al., 1975; Virtanen and Wartiovaara, 1976; Sevaljevic et al., 1979). However in the light of the inefficiency of removal of membrane proteins by nonionic detergents, these results may confirm rather than disprove the presence of membrane components in chromatin. Stein et al.(1975) found that in He La S_3 cells labelled with ^{3}H glucosamine, a large proportion of the radioactivity sedimented with the Triton-washed chromatin and was found to be protein associated on SDS gel electrophoresis. Sedimentation through 1,7M sucrose removed only 10% of the radioactivity. Glucosamine is a major carbohydrate component of the nuclear envelope (Harris, 1978) and nuclei incubated with fluorescein labelled wheat germ agglutinin, a lectin specific for glucosamine are heavily stained only on the inner and outer nuclear membranes (Virtanen and Wartiovaara, 1976). Detergent-washed sea urchin chromatin has been shown to bind the glucose specific lentil lectin (Sevaljevic et al,, 1979). Goldberg et al.(1978) obtained glycoprotein extracts from rat liver chromatin which contained either mannose, fucose or N-acetyl-glucosamine, all well established carbohydrate components of the nuclear envelope (Harris, 1978).

The assumption therefore that if acceptor sites have been located in isolated chromatin, these are necessarily bona fide genomic components and thus able to exert direct effect on processes such as repression and derepression, must be viewed with caution. Until sound methodology is established to ensure that chromatin can be isolated free of membranous and other contaminants, doubts will continue to be raised in respect of the "in vivo" location of acceptor sites found in isolated chromatin.

1.3.2 The Nuclear Envelope

The nuclear envelope is by electronmicroscopic criteria an easily recognizable fraction of the nucleus. It consists of a distinct inner and outer membrane frequently interrupted by the presence of pores - i.e. circular appearing holes in the double membrane which for any particular cell type are of uniform dimension;

generally in the range 40 - 80nm (Aaronson and Blobel, 1975). The outer nuclear membrane has been observed by electron microscopy to be continuous with the endoplasmic reticulum (Franke and Scheer, 1974). There is no evidence that the inner nuclear membrane extends in a similar fashion into the interior of the nucleus. At the pore periphery there appears to be continuity between inner and outer membrane (Franke and Scheer, 1974).

The ultrastructure of the pore complex has been studied extensively and different models proposed (Franke and Scheer, 1974). Interpretations from negatively stained and freeze cleaved material suggest that there are 8 subunits present in the annulus (Harris, 1978). Image enhancement of the pore complex by photographic rotation agrees with this interpretation (Franke and Scheer, 1974). Pores often contain a central granule thought to be a transient ribonucleoprotein particle. They have a characteristic location in the envelope at the distal ends of channels leading through the peripheral heterochromatin (Aaronson and Blobel, 1974). The association of polyribosome chains with the annulus of the pore (Franke and Scheer, 1970) has led to speculation that polyribosome formation may well begin at the outer annulus of the pore complex. A recent paper by Unwin and Milligan (1982) contains some excellent pictures of the pore complexes of Xenopus oocyte nuclear envelopes. The authors suggest that the pore complex is not composed of transmembrane proteins in the usual sense but that the proteins of the complex merely provide a grommet which stabilizes the hole formed by fusion of the inner and outer nuclear membrane.

The function of the pore complex has been the subject of much speculation, particularly in the context of nucleocytoplasmic translocation processes. Franke (1974) has produced a number of remarkable electronmicrographs demonstrating nucleocytoplasmic emission of large material clumps which may contain RNP. They suggest that the pores are not simply gateways for the entry of RNP into the cytoplasm but also sites of final processing and assembly in ribosome formation (Franke and Scheer, 1970). The association of ATPase and RNase with the pores (Zbarsky, 1973) supports that conclusion. Though all observed nuclei have pores, the pore frequency varies considerably. Maul et al.,(1972) have shown that in the lymphocyte system the number of pores per nucleus approximately doubles after phytohemagglutinin stimulation. The increase is biphasic and appears to be related to an increase in the rate of protein- and DNA

synthesis. Nuclear envelope of maturing oocytes of various amphibia contain an unusually high number of pore complexes (Krohne et al., 1978).

Though the structure of the pore complex has been studied extensively, little is known of its composition. Current work suggests that the complex is mainly proteinaceous in nature and consists of 3 or 4 polypeptide chains (Aaronson and Blobel, 1974; Krohne et al., 1978, 1980). Treatment of the isolated envelope or nuclei with a nonionic detergent such as Triton X-100 fails to solubilize or even change the morphology of the pore complexes which remain intact at the nuclear periphery (Aaronson and Blobel, 1974). Clearly the pores do not depend on an intact membrane or the presence of phospholipid for maintenance of their structural integrity. A number of attempts have been made to isolate a homogenous preparation of pore complexes (Harris, 1978; Krohne et al., 1978), but at best so far a pore enriched fraction has been obtained as assessed by electron microscopy (Krohne et al., 1981). At present no methodology exists for the separation of inner and outer nuclear membrane and hence composition of the nuclear envelope must be considered as a whole. The chemical composition of the nuclear envelope has been determined by several groups of workers. The results are summarized below. (From Harris, 1978)

TABLE 1.1

GROSS COMPOSITION OF THE NUCLEAR ENVELOPE

Results expressed as percentage of total mass.

TISSUE	PROTEIN	PHOSPHO-LIPID	DNA	RNA	REFERENCE
Rat Liver	64,0	23,0	8,0	5,0	Agutter, 1972
Bovine Liver	70,4	22,7	1,1	5,8	Berezney et al., 1972
Rat Liver	77,7	16,6	2,0	3,7	Franke et al., 1970
Rat Liver	67,4	26,1	0	6,6	Kashnig and Kasper, 1969

On average the carbohydrate content of the envelope is about 4%.

Stick and Krohne (1982), using Xenopus laevis oocytes, have recently raised antibodies to an envelope protein (mw 68 000) which is the major polypeptide component of the envelope fraction resistant to simultaneous extraction with 1 M KCL and 1% Triton X-100. The antibodies reacted strongly with both the lamina and pore complex. The antigen recognized by these antibodies was not present in the nuclear interior. This result represents the first positive localization of a polypeptide in the nuclear pore complex.

The method chosen for the isolation of the envelope has considerable bearing on the final composition. This is particularly true in respect of the DNA content. Procedures using high salt, sonication or extensive DNase treatment, tend to produce envelopes with a low DNA content. However, these envelopes are often fragmented and cause limited separation of inner and outer membrane. Low ionic strength procedures on the other hand (Kay et al., 1972) have a higher DNA content but maintain superior morphological integrity. Treatment with high salt tends to remove peripheral and loosely bound proteins. The percentage of DNA present in the envelope may thus well be statistical, depending on the method of isolation. However, Agutter (1972) has found that a finite percentage of DNA is necessary for the morphological integrity of the envelope.

Most investigators detect a significant, if low, quantity of RNA attached to the envelope. Scheer (1972) has claimed that most of this is heteronuclear RNA (HnRNA), associated in a transient fashion with the pore complex. However a certain proportion may well be of ribosomal origin. Dabeva et al. (1977) have shown that even detergent purified nuclei retain cytoplasmic ribosomes, normally associated with the outer nuclear membrane.

It is widely accepted that most, if not all, nuclear lipid is located in the nuclear envelope. This is disputed by a few authors (Manzoli et al., 1978) who claim that a small amount of non-envelope lipid is tightly associated with the chromatin. This however, amounts to less than 1% of total nuclear lipid and lipids isolated from whole nuclei are found in virtually the same proportion as in isolated nuclear envelope. The phospholipid content of the nuclear envelope is very similar if not identical to that of the endoplasmic reticulum. Table 1.2 taken from Franke et al. (1976) summarizes phospholipid composition in nuclei, nuclear envelopes, endoplasmic reticulum and plasma membranes from rat liver. Virtanen et al. (1977) confirm these results. No significant amounts of glycolipid have yet been identified in the envelope (Franke et al., 1976).

TABLE 1.2

PHOSPHOLIPID COMPOSITION (MOL % OF TOTAL PHOSPHOLIPID)

	NUCLEI	NUCLEAR ENVELOPES	ROUGH MICROSOMES	PLASMA MEMBRANES
Phosphatidylcholine	55,0	57,7	56,9	39,1
Lysophosphatidylcholine	2,8	2,6	1,4	1,9
Phosphatidylethanolamine	26,4	24,2	26,0	24,0
Sphingomyelin	4,5	5,8	6,5	23,5
Phosphatidylinositol	7,6	6,4	6,9	7,5
Phosphatidylserine	2,5	1,8	2,0	3,5

After Franke et al., 1976

Studies on the protein composition of the nuclear envelope have tended to rely mainly on SDS gel electrophoresis, and the polypeptide distribution obtained varies somewhat depending on the method of isolation. SDS gels show a general sparsity of bands in the high molecular weight region other than a prominent band at approximately 170 000 daltons. A preponderance of polypeptides - up to 50% of total material - is found in the 50 000 - 80 000 dalton region.

The amino acid composition of the envelope protein fraction indicates a preponderance of acidic polypeptides (Bornens and Kasper, 1973). The ratio of acidic to basic residues decreases with decreasing molecular weight of polypeptides. The acidic nature of the envelope polypeptides makes them difficult to distinguish from non envelope acidic chromosomal proteins. Jackson (1976) found that the polypeptides in a preparation of erythrocyte acidic chromosomal protein, are by SDS gel electrophoresis, almost identical to those in a preparation of erythrocyte nuclear membrane. A further complication is the close similarity in the polypeptide composition of nuclear envelopes and microsomal membranes (Atger and Milgrom, 1978; Richardson and Agutter, 1980) making extensive microsomal contamination of an envelope preparation difficult to detect. Franke et al.,(1976)have even suggested that the nuclear envelope must be considered as a highly purified section of microsomal membrane.

Lam and Kasper (1979a) propose that three polypeptides in the range 68 - 78 daltons comprise the structural elements of the pore complex, and that two of the polypeptides exist as homopolymers. One of these polypeptides (mw 68 000) is phosphorylated by an endogenous protein kinase (Lam and Kasper, 1979b). Scheer et al.(1976) on the other hand, using micromanipulator isolated nuclear envelopes from frog oocytes which have an unusually high number of pore complexes, are able to prepare a pore enriched fraction comprising two major polypeptides of 150 and 73 kilodaltons. The same authors observe in rat liver nuclear envelopes two additional components of 77 and 66 kilodaltons. Antibodies raised to the 73 000 molecular weight polypeptide bound specifically to areas in the nuclear periphery although only weakly to pore complexes (Krohne et al., 1978).

The predominant polypeptides - 70, 67 and 60 kilodaltons of the pore lamina complex (Aaronson and Blobel, 1975) are ascribed not to the nuclear pore complex but to the lamina, and have been named Lamins A, B and C (Gerace and Blobel, 1980; Shelton et al., 1980a). Lamin C has recently been shown to be a breakdown product of Lamin A produced after extensive boiling of samples prior to SDS gel electrophoresis (Shelton et al., 1980). Lamins A and B can be crosslinked via oxidation of intrinsic disulphide bonds (Shelton et al., 1982). They suggest that Lamin B occurs as a tetramer in the envelope. Lam and Kasper (1979) have also proposed that these disulphide linked complexes may serve as structural components for the pore complex. These three polypeptides have also been proposed to constitute the major components of the nuclear protein matrix (Berezney and Coffey, 1977). The observed association of a proteinaceous lamina (Aaronson and Blobel, 1975; Shelton et al., 1980a; Gerace and Blobel, 1980) and possibly nuclear protein matrix with the inner nuclear membrane has made the assignment of certain polypeptides to particular substructures such as the pore complex, difficult. The major proportion of polypeptides from the envelope, lamina and matrix all occur in the range 50 000 - 80 000 daltons.

The carbohydrate component of the nuclear envelope has until recently received scant attention. Of the few studies available on the carbohydrate content of the nuclear envelope, the available information suggests that most of the carbohydrate is associated with protein rather than lipid (Franke et al., 1976). Kawasaki and Yamashina (1972) and Franke's group have performed carbohydrate analysis of the rat liver nuclear envelope. The predominant components are mannose, glucose and glucosamine,

with smaller amounts of galactose and galactosamine and virtually no sialic acid, a prominent component of the plasma membrane. As is the case for nuclear lipid, the bulk of total nuclear carbohydrate can be accounted for by the nuclear envelope (Kawasaki and Yamashina, 1972). 100% of mannose and at least 60% of glucosamine were found to be membrane associated. This finding is significant in view of the continuing debate over whether glycoproteins of non membrane origin are components of the eukaryotic genome (Goldberg et al., 1978; Sevaljevic et al., 1979; Stein et al., 1975). Goldberg et al.,(1978) for example, found mannose containing glycoproteins in their chromatin preparation and mouse HMGs 14 and 17 have recently been identified as glycoproteins (Reeves et al., 1981).

A number of groups have shown extensive binding of ConA to the nuclear envelope, confirming the presence of mannose (Virtanen and Wartiovaara, 1976; Virtanen, 1977; Monneron and Segratain, 1974). In keeping with findings on the membrane localization of nuclear mannose, nuclei labelled with fluorescein ConA tend to stain mainly along the membrane. An interesting observation is that this labelling occurs primarily along the cisternal surfaces of both inner and outer membranes (Virtanen and Wartiovaara, 1976). The binding of fluorescein ConA to proteins separated in polyacrylamide gels indicates that all the major proteins of the nuclear envelope contain appreciable amounts of mannose (Virtanen, 1977). Franke et al.,(1976)obtain a figure of 17µg/mg protein for the carbohydrate content of the nuclear envelope although values as high as 40µg/mg protein have been reported (Kashnig and Kasper, 1969).

Part of the difficulty in obtaining chromatin free from nuclear envelope contamination is the close "in vivo" association of the envelope with chromatin fibres. Attachment of DNA to membranes appears to be a phenomenon ubiquitously seen in the biological kingdoms. In many cases, specific functions of this relationship remain to be elucidated. The nature of the attachment has yet to be established. Membrane association of DNA has been observed in prokaryotes, eukaryotes, viruses and extra-chromosomal DNA. A variety of techniques including electron microscopy, autoradiography, selective salt extraction and density gradient ultracentrifugation have been employed to study their association.

In prokaryotes this attachment may provide a structural basis for DNA replication and chromosome segregation"in vivo"(Leibowitz and Schaechter, 1975). Although in prokaryotes the association of DNA with the membrane appears to be a prerequisite for "in vivo" DNA replication, the nature

of the membrane-multi-enzyme complex is not known. "In vitro" the DNA synthesizing proteins of E. Coli are functional in a soluble form (Kornberg et al., 1974).

In eukaryotes, multiple chromosomes and the complexity of the nucleus, preclude a direct comparison to the prokaryotic nucleoid. Many workers, using a variety of cell types from numerous species, have reported an association of chromosomal DNA with the nuclear envelope. Most of the experimental evidence from these studies has been extensively reviewed by Franke (1974).

The association of DNA with the nuclear envelope is well documented. Initial evidence was nearly all electron microscopic (Comings and Okada, 1970a; Comings and Okada, 1970b; Lampert, 1971; Comings and Okada, 1970c). Du Praw (1968) was the first to propose that chromatin forms structural attachments on the inside of the nuclear envelope which may serve to hold interphase chromosomes in fixed positions relative to one another. Quick (1980) observed polytene chromosomes in salivary gland nuclei from chironomid larvae connected along their entire length by some 100 chromatin fibres to the inner nuclear membrane. The condensation of chromosomes to the nuclear envelope during prophase has been clearly demonstrated (Comings and Okada, 1970b).

The general consensus seems to be that the chromatin is anchored at multiple inner nuclear membrane sites but their location relative to the nuclear pore structure remains debatable (Franke, 1974; Ashley, 1974). The DNA-envelope association is stable under a variety of conditions, including ultra-centrifugation, high or low salt concentrations, treatment with urea, detergents or chelating agents. Some DNA is even protected from degradation by DNases due to its close association with the membranes (Franke, 1974).

The functional role of the association is unclear. Especially the functional role during replication is controversial and has been debated by a number of authors (Clay et al., 1975; Cabradilla and Toliver, 1975; Comings and Kakefuda, 1968; Mizumo et al., 1971; Mizumo et al., 1971; Infante et al., 1973; Huberman et al., 1973; Hobart et al., 1977). Most experiments in this area involve the incorporation of ^{3}H thymidine into a synchronous cell population followed by thin sectioning and autoradiography. In general an unsynchronized cell population pulse labelled for five minutes with ^{3}H thymidine will have grains spread throughout the nucleus on EM autoradiography. However Comings and Kakefuda (1968)

found that in a similar experiment using synchronized cells, the grains were localized near the envelope. After a 1-hour pulse chase the membrane associated label moved to the nuclear interior and they concluded that initiation of replication takes place at the membrane whereas replication may occur throughout the nucleus. Huberman et al.,(1973) disputed these results. Using synchronized chinese hamster cells and a 30-second pulse time, since it had been calculated that only 1,25µm of DNA could be synthesized in 30 seconds, they found grains throughout the nucleus in early S phase. Since many of these sites were too far from the envelope to have been initiated there, they concluded that the envelope was not needed for DNA replication.

Hobart et al.,(1977) performed a similar experiment using the sea urchin system in which fertilized eggs are naturally synchronous for the first three divisions. A 30-second pulse in early S phase produced a peripheral labelling pattern. They concluded that the synthesis of DNA begins at the membrane and that newly synthesized DNA is translocated to the central area of the nucleus. They attributed the conflicting results of the other authors to repair synthesis and sporadic bursts of replication found in artificially synchronized cell lines. Other evidence linking newly replicated DNA to the nuclear envelope comes from nuclear fractionation studies using the detergent Sarkosyl to produce a DNA-Sarkosyl-membrane complex which has been named the M band (Sinha and Mizumo, 1977; Clay et al., 1975; Infante et al., 1973). This complex contained the bulk of newly replicated DNA. Certainly the necessity of DNA-membrane association for initiation of chromosomal replication has a precedent in prokaryotic systems (Winston and Sueoka, 1980). Hopefully this matter will soon be settled with regard to eukaryotic systems.

There have so far been no reports on the association of glucocorticoids or glucocorticoid receptor complexes with the nuclear envelope. The association of androgens and estrogens with the envelope has already been referred to (see 1.1).

1.3.3 The Pore Lamina Complex and Nuclear Protein Matrix

In contrast to the nuclear envelope, which is a reasonably well defined subfraction of the nucleus, two other less well defined nuclear subfractions have been reported: the lamina and the nuclear protein matrix (Aaronson and Blobel, 1975; Berezney and Coffey, 1976). Both are proposed to have a close association with the envelope and neither can be isolated without considerable contamination by envelope polypeptides and in particular the pore complex.

The nuclear lamina is identified by electron microscopy as a densely stained layer directly below the envelope. It has been described by various authors (Aaronson and Blobel, 1975; Dwyer and Blobel, 1976) and isolated from a number of sources, but always in association with the nuclear pore complex (Aaronson and Blobel, 1975; Shelton et al., 1980a; Gerace and Blobel, 1980; Dwyer and Blobel, 1976;Gerace et al., 1978). It appears on electron microscopy to be a 15nm thick proteinaceous structure which opposes the inner nuclear membrane, connecting the pore complexes and surrounding the entire nucleus. Aaronson et al. (1975) suggest that the lamina interconnects and orientates the pores.

The general isolation scheme for the pore complex lamina involves prior isolation of the nuclear envelope by conventional methods followed by successive treatments with 2% Triton X-100 and 2M NaCl. According to the authors (Dwyer and Blobel, 1976) this removes both inner and outer membranes and leaves the pores and submembranous lamina. The efficacy of Triton in complete solubilization of either membrane has been queried by numerous authors (Jackson, 1976; Frederiks et al., 1978; Tata et al., 1972). Triton X-100 extraction of purified endoplasmic reticulum membrane which contain no pores or associated lamina also produces a considerable insoluble residue comprising at least 30 discrete polypeptide chains as viewed by SDS gel electrophoresis (Richardson and Agutter, 1980; Agutter and Richardson, 1980). Hence a number of authors (Scheer et al., 1976; Berezney and Coffey, 1976; Comings and Okada, (1976) suggest that the lamina may possibly represent a collapsed envelope structure or altered inner nuclear membrane. It has recently been proposed that the lamina forms a shell around the nucleus which can disintegrate and reform during the cell cycle (Gerace and Blobel, 1980).

A detailed outline of the isolation, structure and properties of the nuclear matrix isolated from rat liver nuclei has been presented by Berezney and Coffey (1976). The structure appears to consist of three electron microscopically visible main components. 1. A residual nuclear envelope with readily visible pore complexes 2. Highly condensed residual nucleoli 3. An extensive granular and fibrous matrix structure which extends throughout the nucleus. Prior to the DNase digestion step

in the isolation procedure, DNA fibrils can be observed to form an intricate meshwork structure in close association with the matrix (Berezney and Coffey, 1976; Long et al., 1979). At present it is unknown if the matrix is a continuous"in vivo"structure; however its functions and role have been speculated on by a number of authors (Berezney and Coffey, 1976; Berezney et al., 1979; Berezney and Coffey, 1977; Long et al., 1979). Apart from reaching the conclusion that an intranuclear matrix may confer a degree of structural organization on the nucleus, Berezney has observed the association of newly replicated DNA with the matrix (Berezney and Coffey, 1976; Berezney et al.,1979 , Berezney and Buchholtz, 198la,b) and has suggested that extranucleolar RNA synthesis may be associated with it. Globin messenger RNA coding sequences have been found associated with the matrix of duck erythroblasts (Maundrell et al., 1981). Robinson et al.,(1982) have found that the ovalbumin gene is associated with the nuclear matrix of chicken oviduct cells.

Wunderlich et al.,(1978) has observed that nuclear matrices have the ability to expand and contract under the influence of divalent cations, indicating the presence of contractile proteins. The matrix also binds 17-β-estradiol (Agutter and Birchall, 1979; Barrack and Coffey, 1980) and is associated with heteronuclear RNA (van Eekelen and van Venrooij,1981. The similarities between the pore lamina complex and the nuclear protein matrix have been noted (Berezney and Coffey, 1976). A possible reason for this becomes evident if one considers the isolation procedure for the matrix consists of successive extraction of nuclei with 0,2mM $MgCl_2$, 2M NaCl and 1% Triton X-100 followed by a final incubation with DNase and RNase (Berezney and Coffey, 1976). In the case of the pore lamina isolation this procedure is essentially reversed - liberation of the envelope with DNase and RNase and subsequent treatment with Triton X-100 and 2M NaCl (Aaronson and Blobel, 1975). The chemical composition of matrix and lamina are similar (Table 1.3).

Furthermore on SDS gel electrophoresis the three major polypeptides which account for between 25% and 35% of the total protein appear very similar: 66 - 69 kilodaltons for the lamina (Aaronson and Blobel, 1975) and 62 - 69 kilodaltons for the matrix (Berezney and Coffey, 1977). These proteins occur in similar proportions in the nuclear envelope (Franke et al., 1976). The uniqueness of each structure is based mainly on electron microscopic evidence. The matrix is a preparation of preserved nuclear spheres which collapse under high G forces (Berezney and Coffey, 1976) and then appear similar to the collapsed envelope structure of the pore lamina complex. (See also section 1.3.2.).

TABLE 1.3.

PERCENT COMPOSITION OF THE NUCLEAR PROTEIN MATRIX AND PORE LAMINA COMPLEX

	PROTEIN	DNA	PHOSPHOLIPID	RNA	CARBOHYDRATE	SOURCES
Matrix	97,6	0,1	1,1	1,2	5,5	Berezney & Coffey (1977)
Lamina	95,0	3,0	0	2,0	Not done	Dwyer & Blobel (1976)

Berezney (1977) notes the persistence of nuclear envelope polypeptides in the matrix and admits that the three major polypeptides of the matrix may well be pore complex components. The matrix also binds lectins. The number of lentil lectin binding sites on nuclear matrix isolated from sea urchin embryos was of the same order as the number found in intact nuclei (Sevaljevic et al., 1981). Lectin binding by the nuclear envelope is well documented (Virtanen, 1977). A high molecular weight (174 kilo-daltons) glycoprotein has been identified in matrix fractions from Drosphila and rat liver (Fisher et al., 1982). A glycoprotein of similar molecular weight has been found in nuclear envelope fractions from the livers of rats, guinea pigs, opossums and chickens. The proteins components of the residual nucleoli which co-isolate with the matrix have recently been identified by Franke et al.,(1981). An unsuccessful attempt has been made to fractionate the rat liver nuclear matrix into morphologically distinct components (Kuzmina et al., 1981). The authors noted the persistent insolubility of the pore complex after treatment with either alkali or EDTA.

The matrix may be an artifact produced during one of the nuclear extraction steps, as not all procedures which disrupt the nucleus give rise to a matrix. Giese et al.,(1980) find that treatment of nuclei from Tetrahymena with DNase and RNase followed by extraction with 1M NaCl completely removes the nuclear content and leaves only nuclear envelope ghosts in high yield. Treatment of whole nuclei with the polyanion heparin (Bornens and Courvalin, 1978) produces only one well defined insoluble fraction , namely the nuclear envelope. Gerace and Blobel (1980) are able to completely remove the

contents of their nuclei by successive treatment with DNase, RNase and 0,5M KCl. Agutter and Richardson (1980) have recently reviewed the composition, structure and function of the matrix and other nuclear non-chromatin proteinaceous structures. The isolation methods used in the preparation of rat liver nuclear matrices, nuclear envelopes and pore complex laminas have also recently been investigated in detail (Kaufmann et al.,1981). They demonstrate that seemingly slight changes in isolation procedures cause major changes in the morphology of the residual structures. By varying the order of extraction steps and the extent of disulphide cross-linking, they obtained from a single batch of nuclei, residual structures differing widely in morphology and composition.

It appears that the isolation of either the lamina or the matrix is highly dependent on the inclusion at some stage during the isolation of extraction with a nonionic detergent. It is notable that workers who omit this step but retain those employing high salt and DNase are unable to produce a matrix structure. Nuclei washed with Triton X-100 and subsequently treated with heparin give rise to structures resembling extracted nuclei which Adolph (1980) has termed nuclear scaffolds. These have a similar polypeptide distribution to the matrix as revealed by SDS gel electrophoresis. Nuclei not previously treated with Triton give rise to only the nuclear envelope after heparin treatment (Bornens and Courvalin, 1978).

Significantly, Adolph (1977) has found that chromosomes treated with Triton X-100 and dextran sulphate also give rise to an insoluble protein scaffold similar in appearance to the matrix. These findings have recently been disputed by Okada and Comings (1980), who suggest that the scaffold is an artifact. Detke and Keller (1982) compared the proteins of HeLa cell nucleoskeletons and chromosome scaffolds by two-dimensional electrophoresis and peptide mapping. They found the major proteins of each fraction to be identical. The limited solubility of nuclear envelope polypeptides in Triton X-100 has already been referred to (1.3.1.2). Thus both matrix and lamina, which already contain the envelope pore complexes, may also contain a substantial amount of collapsed envelope. From the quantitative point of view, the bulk of lamina and matrix material may well derive from the envelope. Since undoubtedly the natural environment of proteins in the envelope is a hydrophobic lipid bilayer, removal of the latter must lead to an aggregation of the former and attachment to other hydrophobic proteins viz the pore complex. If this is the case, the biological significance of both structures must be called into question.

1.4 SCOPE OF THIS THESIS

Although many workers have suggested that chromatin or DNA provide the final acceptor sites for steroid hormone-receptor complexes, it is clear that the complex must at least negotiate the nuclear envelope if its final destination is in fact an intranuclear one. The pore complex is assumed by most authors to be the point of entry of the steroid hormone-receptor complex into the nucleus. The nuclear envelope clearly must have a role that is greater than only compartmentalization of nuclear material. The envelope is in a unique position to receive, mediate or orientate cytoplasmic signals destined for the nucleus. The attachment of DNA at numerous places along the inner nuclear membrane makes this membrane's involvement as a possible mediator of cytoplasmic signals quite feasible. Both insulin (Vigneri et al.,1978) and neural growth factor (Yanker and Shooter, 1979) have been shown to bind to the nuclear envelope, and this binding may well mediate their long term effects on gene expression in target cells.

If the proposed mechanism of steroid hormone action as outlined in Figure 1.1 represents an extreme simplification of the stoichiometric model, then the mechanism outlined in Figure 1.2 can be seen to represent the extreme simplification of the catalytic model, whereby cytoplasmic signals (e.g. steroid hormone-receptors) interact with the nuclear envelope, initiating a catalytic response leading to protein modification and increased or decreased genetic activity. Histones are obvious candidates for modification. The final mechanism of steroid action may well involve elements of both models with the response being mediated both catalytically and stoichiometrically.

In the work presented in this thesis, the interaction of partially purified cytoplasmic steroid hormone-receptor complex with the nuclear envelope is investigated and an attempt made to identify the location of acceptor sites in the membrane. The relationship between the nuclear envelope and nuclear protein matrix is also briefly considered.

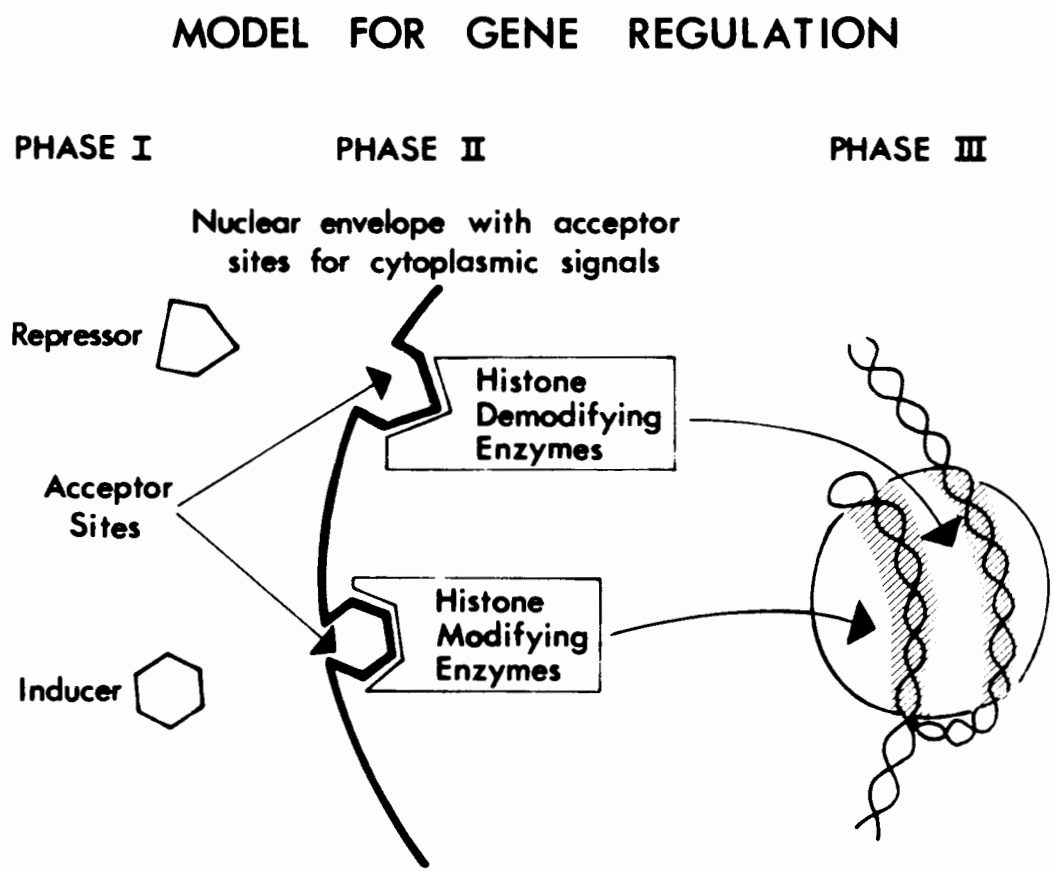

FIGURE 1.2 Catalytic model for interaction of cytoplasmic signals with the nucleus.

PART 2

ISOLATION AND CHARACTERIZATION OF THE ACTIVATED TRIAMCINOLONE-ACETONIDE (TA) RECEPTOR COMPLEX FROM RAT LIVER CYTOSOL

INTRODUCTION

The basic methodology chosen for the isolation of the cytosolic TA-receptor complex from rat liver is that developed by Climent et al.(1977) which utilizes the fact that the nonactivated steroid hormone-receptor does not bind to phosphocellulose whereas it is tightly bound after activation. Their method was effective in separating the activated cytoplasmic triamcinolone acetonide (TA)-receptor complex from other cytosolic steroid binding proteins and the final preparation appeared to contain only one steroid binding protein. Although this method achieved a 3 300 fold purification of the receptor, the preparation was only 10% pure with respect to homogeneity. Westphal and Beato (1980) extended the purification scheme to obtain a homogenous preparation of TA-receptor complex but with a five fold decrease in yield.

For the purpose of this study it was necessary to obtain an activated steroid hormone-receptor complex free of any other cytosolic steroid binding proteins. As this was achieved by Climent et al. (1977) even though their preparation was contaminated by non steroid binding proteins, their procedure was followed.

2.1 Isolation

The isolation of the activated cytoplasmic TA-receptor complex is outlined in detail in 7.2.9. The isolation procedure is outlined in Figure 2.1. The results are summarized in Table 2.1. The charged cytosol was passed through the first and second phosphocellulose columns as quickly as

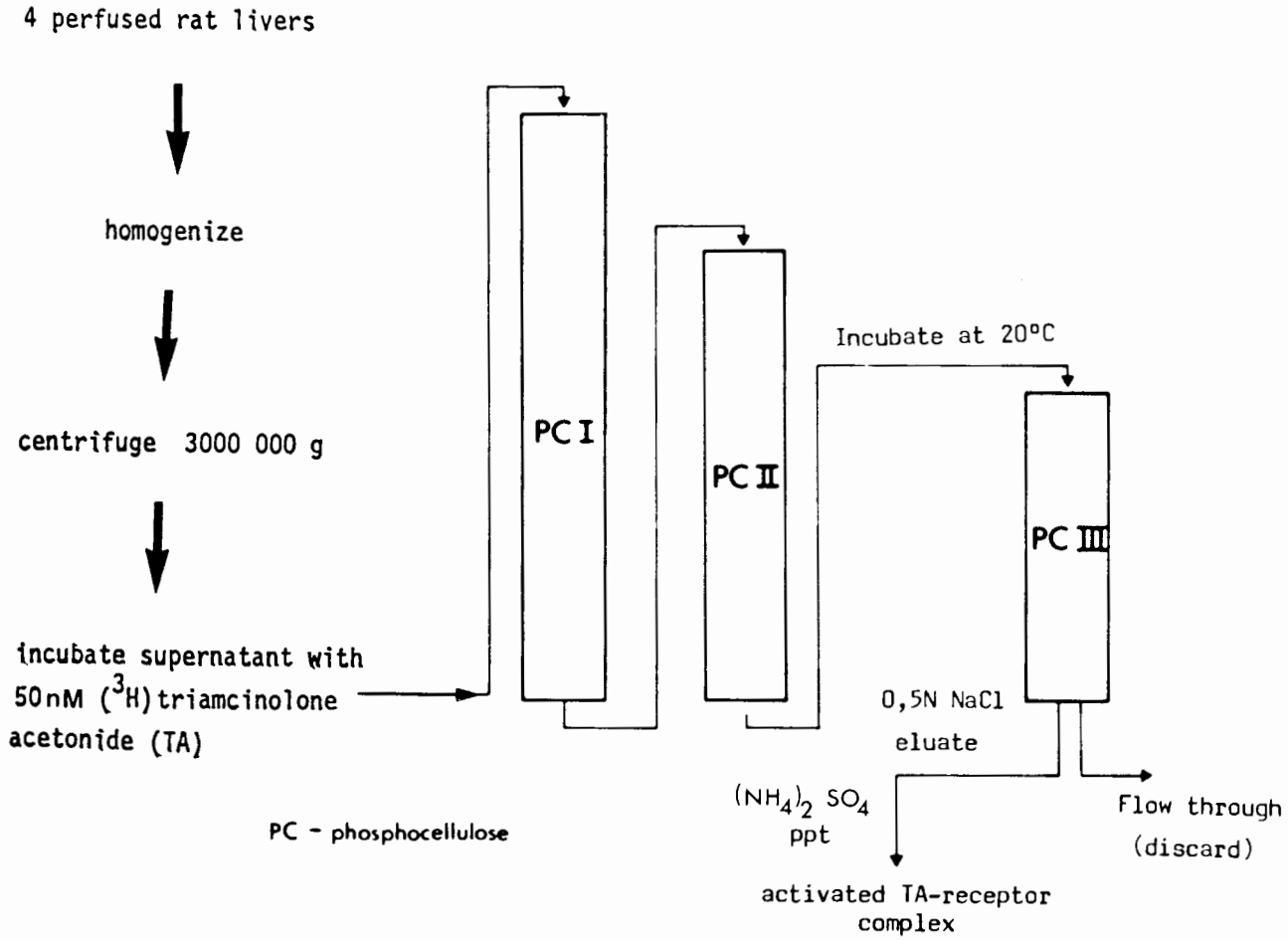

FIGURE 2.1 Schematic outline of isolation of TA-receptor complex.

possible ($\pm$ 3 mls/min.) as prolonged exposure to phosphocellulose may activate the TA-receptor complex (Climent et al., 1977). About 70% of protein and 50% of radioactivity was recovered after passage over the first two columns. Elution of the activated complex from the third phosphocellulose column yielded just 0,01% of original protein and 2,2% of radioactivity. The final ammonium sulphate precipitation served both to concentrate the complex and produce a further tenfold purification. The protein yield of TA-receptor complex in the final step was sometimes below the limits of detection by the Folin Lowry reaction and the final value for purification given in Table 2.1 represents the lower rather than upper degree of purification achieved in a typical isolation. Occasionally difficulties were experienced in the resolubilization of ammonium sulphate precipitated material. The tendency for the activated hormone-receptor complex to aggregate nonspecifically with other cytosol proteins has been noted by other workers (Climent et al., 1976). Because of this aggregation, the final precipitation step was sometimes omitted and the column 3 eluate was diluted to 0,1M NaCl and used. The yield per isolation varied from between 25 to 50 p mole of triamcinolone-receptor complex, assuming 1:1 stoichiometry between hormone and receptor.

Unlabelled steroid hormone-receptor complex was required for competition experiments. However, it was not possible to prepare an unlabelled preparation, as the purification of the complex could only be monitored by inclusion of a radioactive steroid. "Unlabelled" receptor-complex was therefore prepared by using triamcinolone acetonide with a specific activity of one tenth - 0,7 Ci/nmole - sufficient to monitor the purification steps.

TABLE 2.1

PURIFICATION OF CYTOPLASMIC TRIAMCINOLONE-ACETONIDE RECEPTOR

FRACTION	VOLUME ml	TOTAL PROTEIN mg	TOTAL RADIO-ACTIVITY dpm	dpm/mg PROTEIN	PURIFI-CATION	YIELD (RADIO-ACTIVITY) %
Cytosol	31,0	796,7	$4,005 \times 10^7$	$5,026 \times 10^4$	1,0	100,0
Col. 1 flow through	33,0	676,5	$2,780 \times 10^7$	$4,122 \times 10^4$	0,82	67,6
Col. 2 flow through	36,0	567,0	$2,260 \times 10^7$	$3,984 \times 10^4$	0,79	56,4
Col. 3 eluant (0,5M NaCl)	16,5	0,0825	$8,968 \times 10^5$	$1,087 \times 10^7$	216,25	2,23
Ammonium sulphate precipi-tate	0,5	0,005	$6,169 \times 10^5$	$1,240 \times 10^7$	2466,00	1,53

These results represent the average of four isolations.

2.2 CHARACTERIZATION

The S value of the activated complex was determined by comparison with standards of known molecular weight (7.3.4). Samples were run on linear 5 - 20% sucrose gradients for 19 hours with myoglobin and ovalbumin as standards. For these, S values of 2,07 and 3,55 respectively have been reported (C.R.C. Handbook of Biochemistry, 1968). The results are shown in Figure 2.2. The activated TA-receptor complex has an S-value of 2,5 - 3,5S, similar to that found by Climent et al. (1977). Nearly all radioactivity in the gradient was associated with a single peak indicating the presence of probably only a single steroid binding protein. The absence of radioactivity at the top of the gradient indicates that very little free steroid is present. The TA-receptor complex was stored in liquid nitrogen and was viable for several months. The extent of dissociation of steroid from the receptor following freezing and storage was periodically monitored by chromatography on Sephadex G25. A typical elution profile is shown in Figure 2.3. Even after three months storage no more than 3% dissociation of steroid from the receptor was observed. Climent et al. (1977) have shown that the presence of 1 mg/ml albumin is essential if the receptor complex is to remain viable during storage.

Rechromatography of the isolated TA-receptor complex on a small phosphocellulose column resulted in approximately 50% of the preparation being rebound. Clearly, manipulations of the complex after isolation lead to a percentage of the complex becoming "inactive" - i.e. unable to rebind a polyanion such as phosphocellulose. The percentage of "active" steroid receptor present in each batch of hormone receptor varied between 40 and 60%. The "inactive" fraction was unable to bind either nuclei or nuclear envelope.

The extent of aggregation and nonspecific precipitation of TA-receptor complex which had been frozen was also routinely monitored by centrifugation at 50 000g for 30 minutes. Preparations which had undergone ammonium sulphate precipitation during isolation had nonspecific precipitates which varied from between 10 and 60% of total radioactivity. In preparations where the ammonium sulphate precipitation had been omitted, nonspecific aggregation was never more than 15% of total radioactivity.

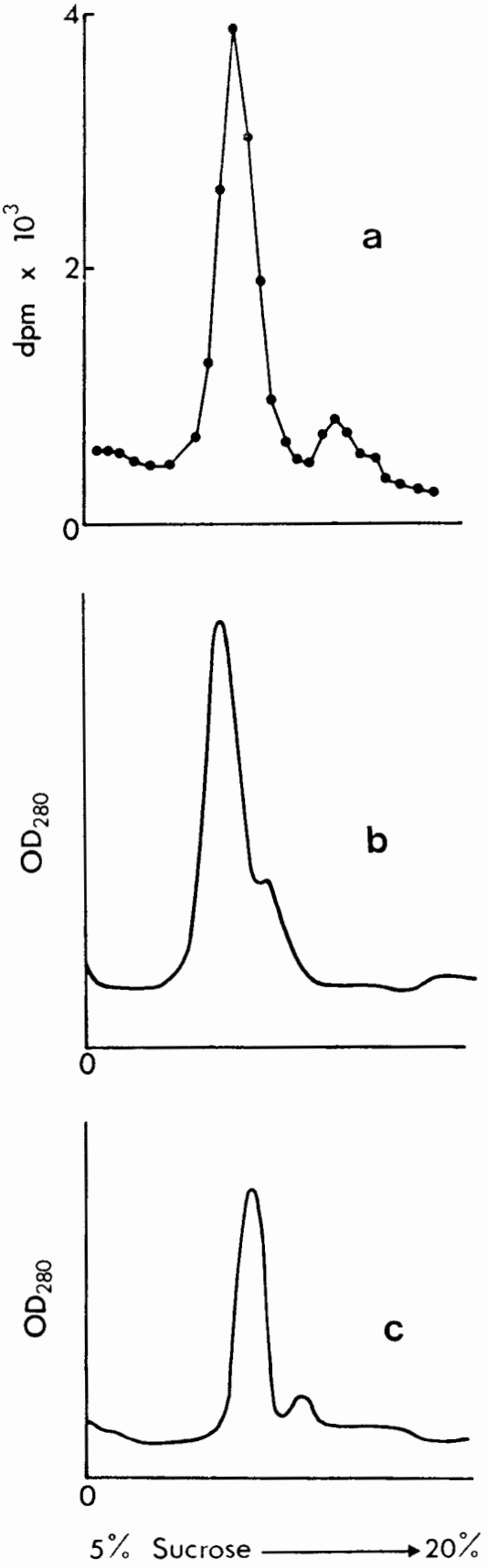

FIGURE 2.2 Centrifugation of TA-receptor complex and protein standards on 5 - 20% linear sucrose gradients for 19 hours as described in 7.3.4.

(a) TA-receptor complex

(b) Ovalbumin

(c) Myoglobin

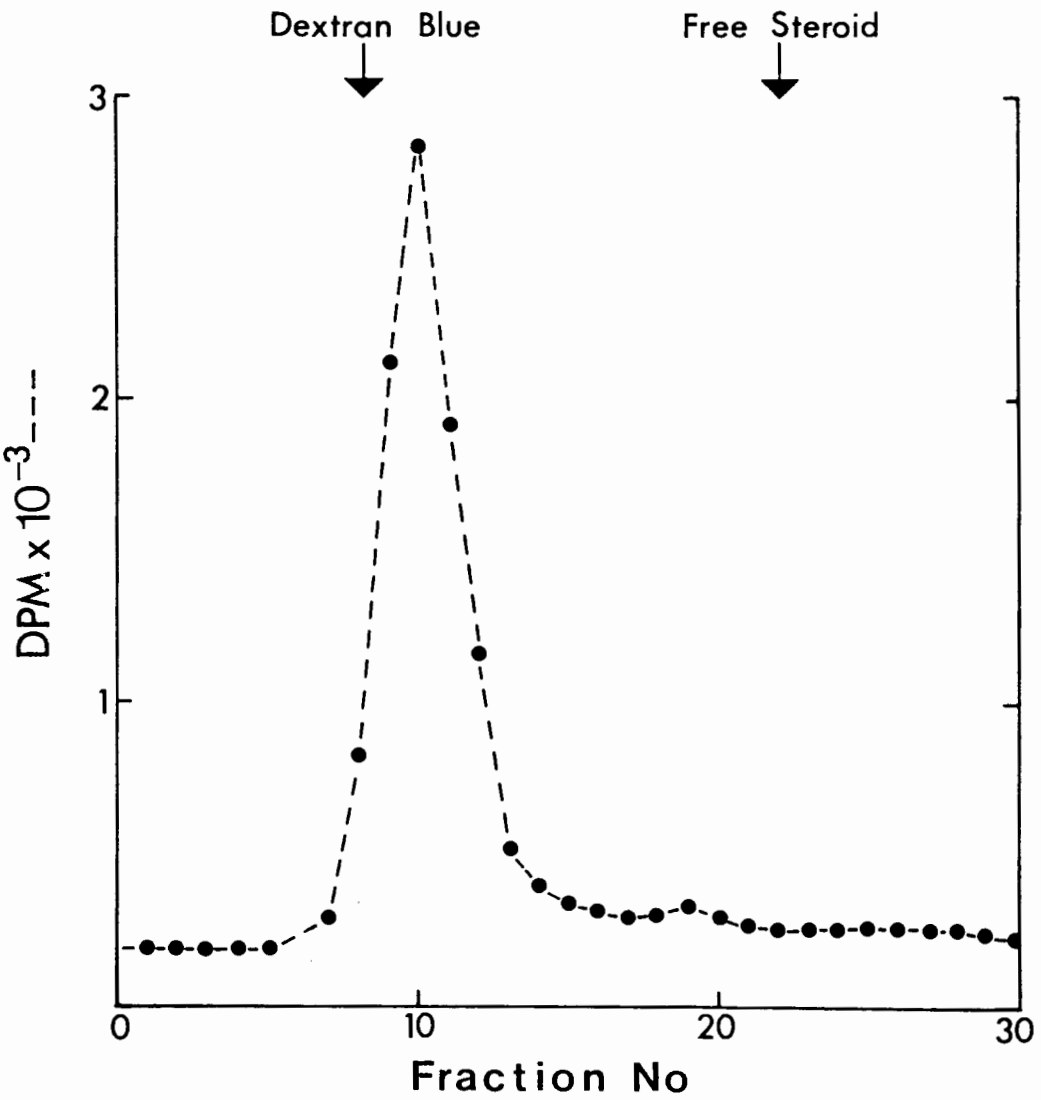

FIGURE 2.3 Chromatography of TA-receptor complex on Sephadex G25 packed in a pasteur pipette. 0,1ml samples were collected and assayed for radioactivity.

Arrows indicate the position of elution of dextran blue and free steroid.

PART 3

LOCALIZATION OF THE NUCLEAR ACCEPTOR SITE FOR TRIAMCINOLONE ACETONIDE-RECEPTOR COMPLEX: ISOLATION OF NUCLEI AND SUBFRACTIONS OF THE NUCLEUS

INTRODUCTION

Although the primary aim of this study was to investigate the interaction of glucocorticoid-receptor complex with the nuclear envelope, it was necessary to check binding of the complex to other nuclear and cellular subfractions for comparative and control purposes. The rat liver plasma membrane has no direct association with the nuclear envelope, while the membranes of the endoplasmic reticulum are continuous with the outer nuclear membrane . These two membrane types were therefore isolated to check the specificity of binding of the TA-receptor complex to the nuclear envelope.

Two nuclear subfractions, chromatin and the nuclear protein matrix, have both been implicated in the nuclear binding of steroid hormone-receptor complexes. These two fractions can also not normally be isolated without considerable contamination by fragments of the nuclear envelope or by nuclear envelope polypeptides. It was therefore considered useful to investigate not only the binding of TA-receptor complex to these fractions but also the extent to which they were contaminated by envelope derived components.

The choice of rat liver as starting material for the isolation of nuclei, nuclear envelope and the glucocorticoid receptor complex was prompted by two factors. In the first place, the liver is a prime target for glucocorticoids. The steroid increases glucose production in the liver, due mainly to induction of gluco-*neogenic* enzymes. Secondly the isolation of virtually every subfraction of the liver cell has been well described. Liver nuclei in particular may be obtained in high yield and purity with ease. Nuclei are comparatively large and therefore a good source of nuclear envelope. Plasma membranes and rough and smooth endoplasmic reticulum are also easily isolated from rat liver in good yield and reasonable purity.

3.1 NUCLEI

3.1.1 Isolation of Nuclei

As starting material for envelope isolation, nuclei needed to be as free as possible of contamination by cytoplasmic or plasma membranes. The use of nonionic detergents for removal of contaminant membranes had to be avoided as these also disrupt the nuclear envelope. The method of Blobel and Potter (1966) which involves sedimentation of nuclei through a final concentration of 2,1 M sucrose provides nuclei in high yield and purity. These authors have observed that a single sedimentation through 2,1 M sucrose produces nuclei with a readily discernable double membrane and little visible contamination by endoplasmic reticulum. Repeated sedimentation of nuclei through heavy sucrose will however shear off the outer nuclear membrane and reduce the yield of envelope. A modification of this method was therefore used for preparation of nuclei.

Rat liver nuclei were isolated as described in 7.2.1. The method employed for the isolation of nuclei avoided the use of nonionic detergents which remove much of the membrane lipid. It relied on the spin through 2,3M sucrose to remove organelles and membranes of a non nuclear origin. Purified nuclei were obtained as a milky white pellet and appeared free of cells and debris when examined by phase contrast microscopy (Figure 3.1.). Suspended in 2,3M sucrose, the nuclei remained intact and did not clump. Clumping is usually a sign of extrusion of chromatin. If the sucrose concentration was reduced to 0,25M, some clumping was observed due to burst nuclei. The yield of nuclei expressed in terms of DNA was in the order of 10mg DNA per 5g rat liver.

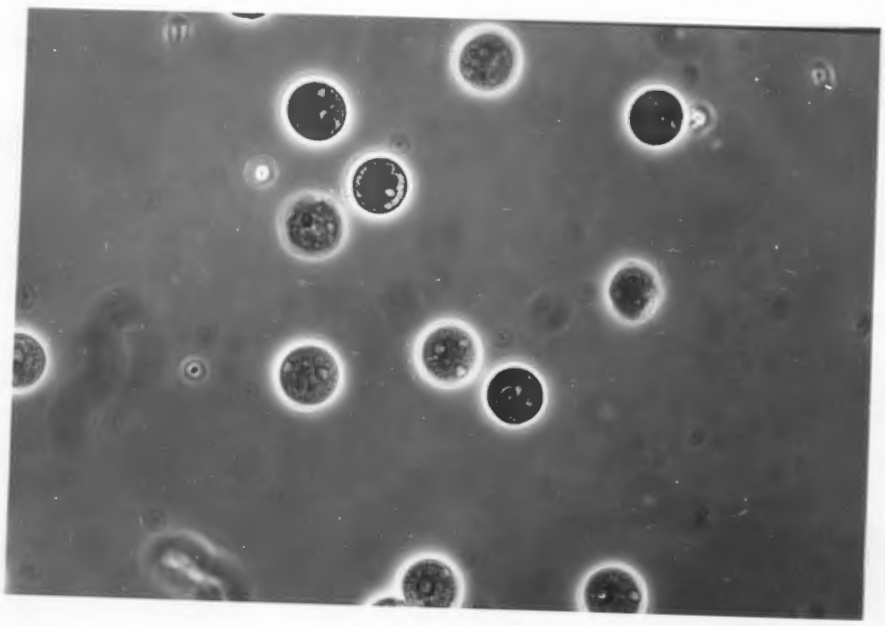

FIGURE 3.1 Purified nuclei suspended in 0,25M sucrose TKM.

3.2 NUCLEAR ENVELOPE

3.2.1 Isolation of Nuclear Envelopes

The isolation of nuclear envelopes is described in detail in 7.2.2. Nuclei were suspended in 0,25M sucrose, 2mM sodium phosphate pH 7.85. Heparin was added to give a final DNA:heparin ratio of 1:1. This resulted in almost immediate solubilization of chromatin with accompanying increase in viscosity of the solution and release of nuclear envelopes. A pellet of crude nuclear envelopes was obtained after centrifugation, and the supernatant saved for further analysis. The pellet sometimes contained a small amount of non-solubilized chromatin in addition to the envelopes. This was effectively removed in the final purification step on a 25 - 50% sucrose gradient. The envelopes formed a sharp band (Figure 3.2) at their characteristic density of 1,18 - 1,20 (Kashnig and Kasper, 1969; Bornens and Kasper, 1973) while any non-solubilized chromatin pelleted. The envelopes were removed by aspiration and if not used immediately, were stored at -170^{o}C.

Occasionally two discrete bands or one of significantly higher density were observed on the gradient (Figure 3.3). These were generated when the concentration of DNA in the heparin solution exceeded 200µg/ml resulting in an excessively viscous solution. This increased viscosity resulted in gross contamination of crude nuclear envelopes with non-solubilized chromatin which persisted in the gradient step, occasionally causing the envelopes to pellet even through 50% sucrose. Incubation of the envelopes with a further amount of heparin effectively solubilized the chromatin. Bornens and Courvalin (1978) have also observed multiple bands on a gradient due to incomplete chromatin solubilization. The total yield of nuclear envelope recovered represented 2,4% of total nuclear protein. Approximately 1 mg of nuclear envelope was obtained from ten grams of rat liver.

3.2.2 Characterization of Nuclear Envelopes

3.2.2.1 Composition

The composition of the purified envelope preparation is given in

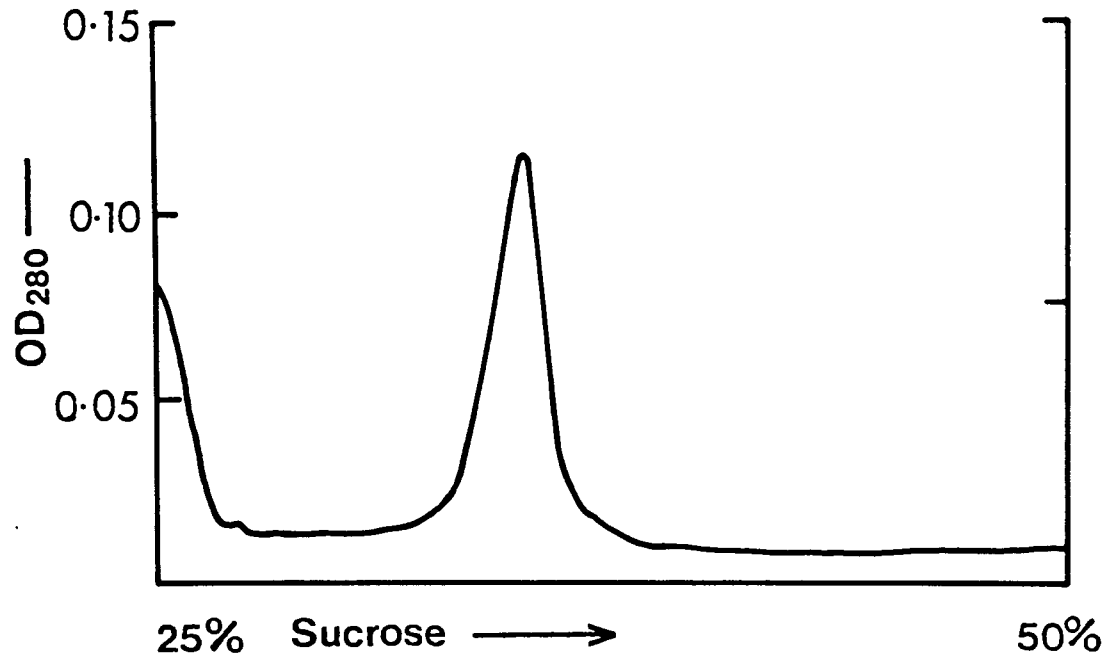

FIGURE 3.2 Sedimentation diagram of nuclear envelopes centrifuged for 3,5 hours at 170 000g in a 25 - 50% sucrose gradient as described in Materials and Methods (7.2.2).

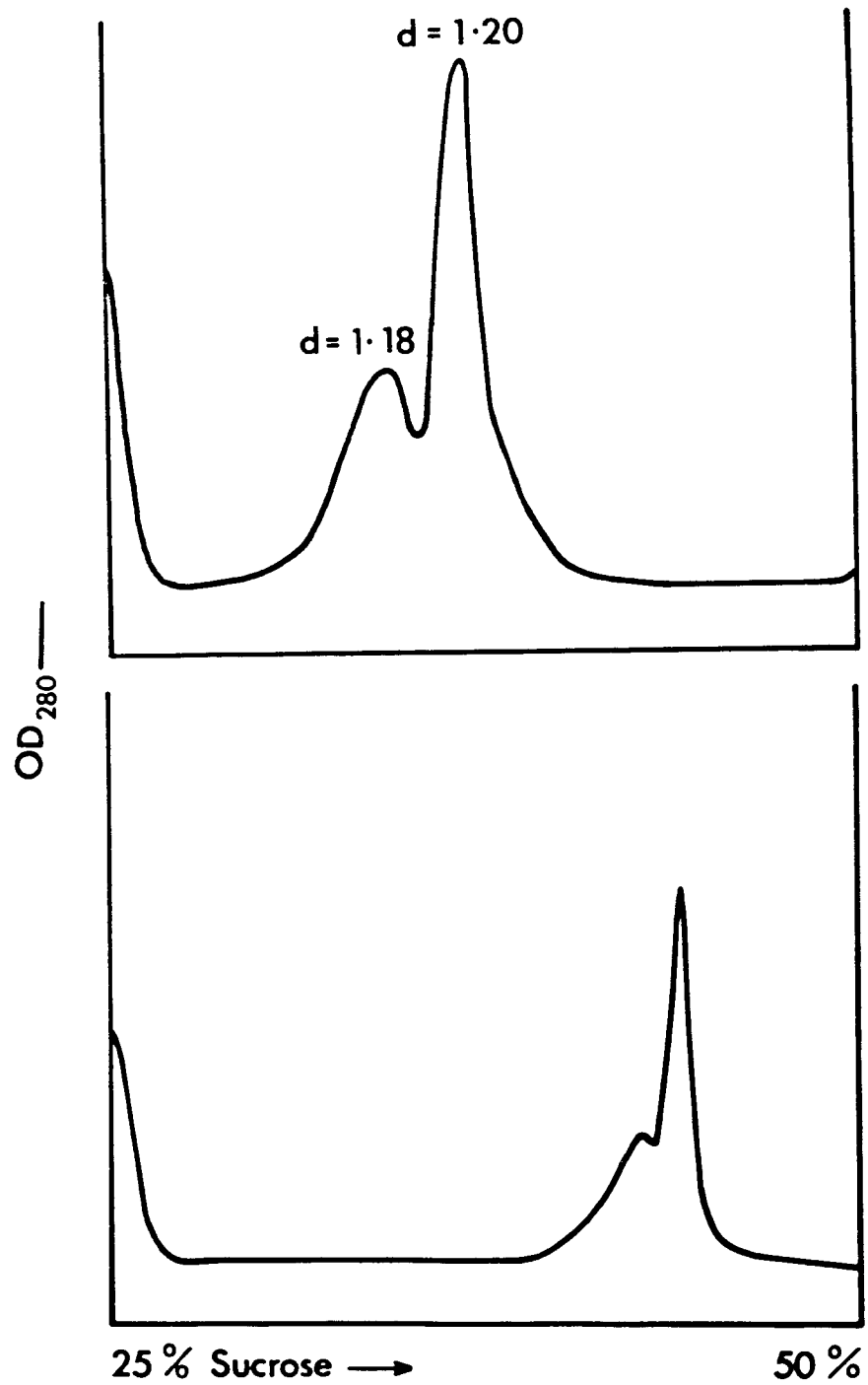

FIGURE 3.3 Sedimentation diagrams of nuclear envelopes contaminated with non-solubilized chromatin. Centrifugation conditions as described in Figure 3.2.

Table 3.1. The results are in good agreement with those of other authors (Kashnig and Kasper, 1969; Harris, 1978; Franke et al., 1976). Less than 0,5% of total DNA was recovered in association with the envelope. The DNA content of the envelope was between 1 and 4% of total envelope mass. Nuclear envelope was solubilized in 2% SDS and scanned continuously over the range 220 - 300nm (Figure 3.4). The UV spectrum shows a peak at 278nm, confirming the predominance of protein as major component in the preparation.

TABLE 3.1

COMPOSITION OF NUCLEAR ENVELOPE

Values expressed as a percentage of total mass.

PROTEIN	PHOSPHOLIPID	CARBOHYDRATE	DNA
60,5 $\pm$ 3,0	32,0 $\pm$ 3,0	3,2 $\pm$ 1,6	2,3 $\pm$ 1,8

Determination of protein, phospholipid, carbohydrate and DNA is described in Materials and Methods.

It is well established that the morphological integrity of the nuclear envelope depends on the intactness of a small amount of bound DNA (Peterson and Berns, 1978; Agutter, 1972). Incubation of nuclear envelope with DNase 1 released only a fraction of bound DNA after 30 minutes incubation at +4^{o}C. Longer incubation with DNase 1 at +4^{o}C or incubation at +25^{o}C for 15 minutes resulted in significant disintegration of the envelope and release of protein and DNA into solution. Such DNase treated envelope no longer sedimented as a sharp band at d = 1.18 on a 25 - 50% sucrose gradient but tended to form a broader, more diffuse zone (Figure 3.5).

3.2.2.2 SDS Gel Electrophoresis

The polypeptide composition of the envelope revealed by SDS gel electrophoresis is shown in Figure 3.6 (a) . The pattern is similar to that

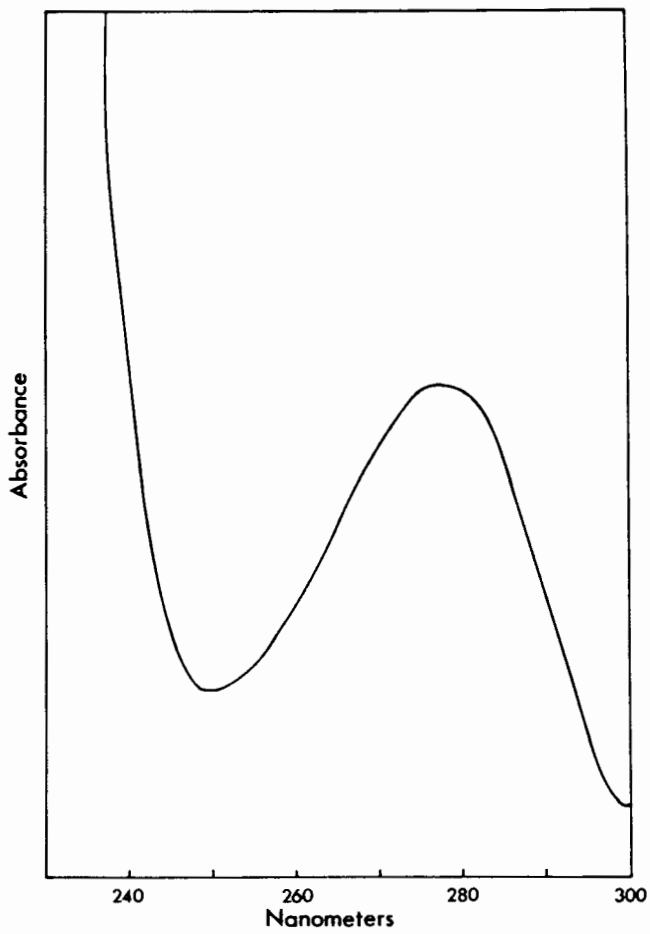

FIGURE 3.4 UV spectrum of total nuclear envelope solubilized in 2% SDS.

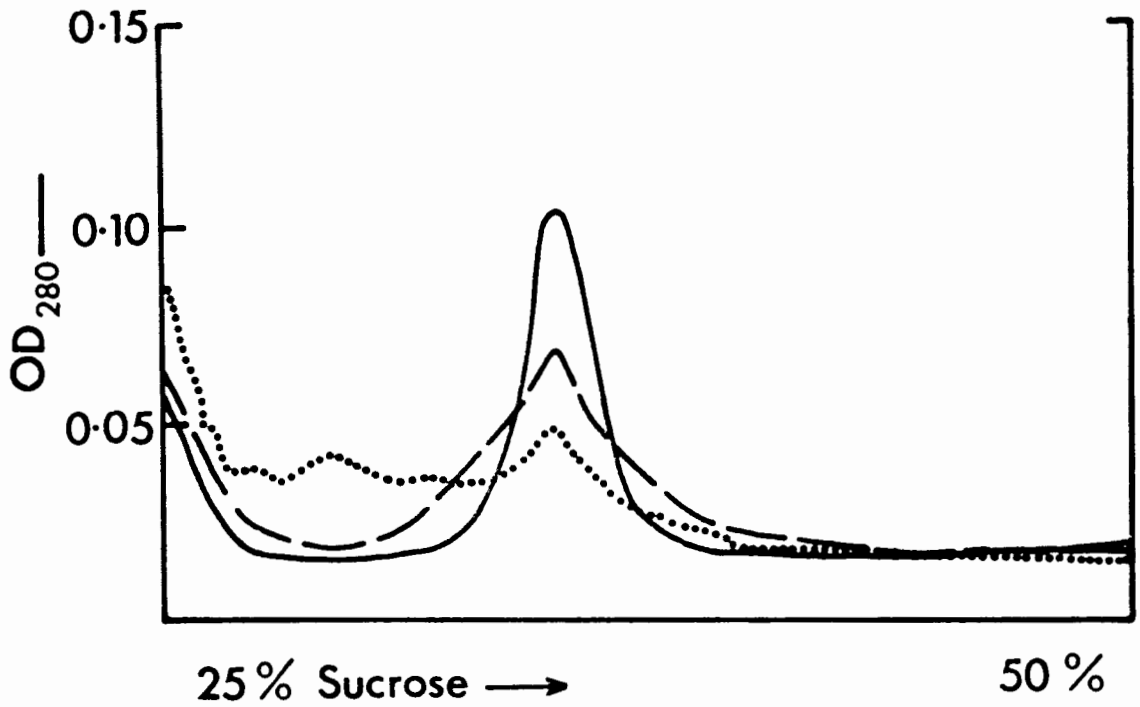

FIGURE 3.5 Sedimentation diagrams of nuclear envelope incubated with DNase

(———) OD_{280} incubated with DNase for 30 minutes at +4°C

(— —) OD_{280} incubated with DNase for 1 hour at +4°C

(.....) OD_{280} incubated with DNase for 30 minutes at +4°C

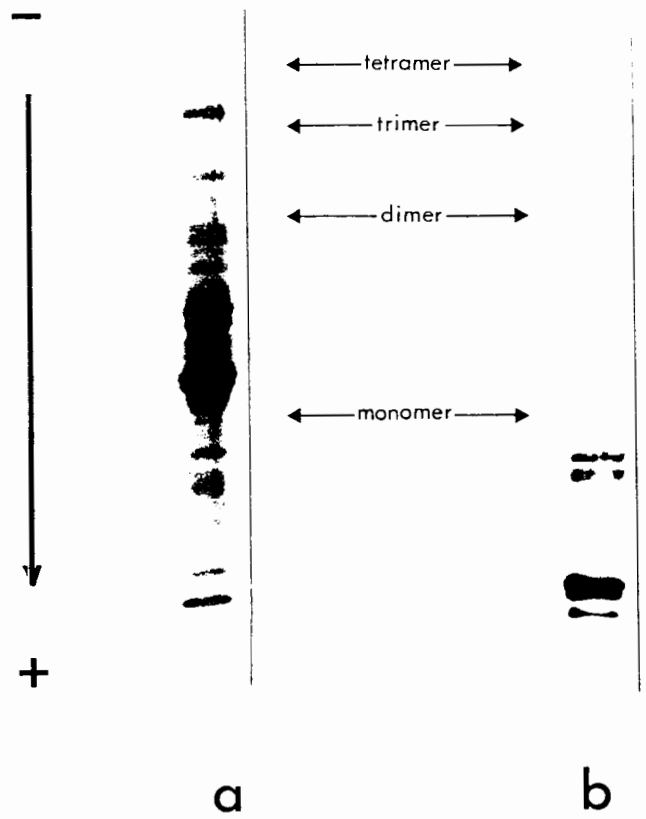

FIGURE 3.6 SDS gel electrophoresis of nuclear envelope (a) and total histone (b) from rat liver. Conditions of electrophoresis are described in Materials and Methods (7.3.1).

Arrows indicate molecular weight marker proteins: monomer = 53 000 daltons (BDH Product No. 44230)

found by other authors (Aaronson and Blobel, 1975; Jackson, 1976; Bornens and Kasper, 1973). Particularly prominent is a high molecular weight band (± 170 kilodaltons) and a triplet at approximately 55 - 60 kilodaltons which together account for 25 - 35 percent of the total Coomassie stained material. Up to 25 other distinct polypeptide chains may be distinguished. The polypeptide composition did not vary from batch to batch of nuclear envelope preparations. A small amount of low molecular weight material migrating with the mobility of histones was found in all preparations (Figure 3.6 (a)). This was expected as the envelope contains a small amount of DNA. However not all the low molecular weight bands migrate with histones as is evident when this region is compared to isolated rat liver histones (Figure 3.6 (b)).

3.2.2.3 Glycoprotein Composition

Fluorescein labelled lectins or a recently developed sensitive silver staining technique were used to detect the presence of glycoproteins after SDS gel electrophoresis. Glycoproteins have usually been identified after gel electrophoresis via the periodic-acid-Schiff reaction (P.A.S.) (Segrest and Jackson, 1972). Recently the staining of glycoprotein with Alcian Blue (Wardi and Michos, 1972) and DANSYL-hydrazine has been introduced (Eckhardt et al, 1976). However the detection limit of the P.A.S. reaction and the Alcian Blue reaction is approximately 2 - 4 µg carbohydrate and whereas the DANSYL reagent may detect approximately 60 ng carbohydrate, the reaction involves the use of dimethyl sulfoxide requiring specific laboratory precautions because of its toxicity. For these reasons, a more sensitive and much simpler method of glycoprotein detection in gels was needed.

Concanavalin A (Con A) and Lens culinaris hemagglutinin (LCH) were isolated and labelled with fluorescein isothiocyanate (7.2.8.2). Both lectins bind glucose and mannose. Con A shows a higher specificity for mannose and its derivatives (Agarwal and Goldstein, 1967) while LCH has a higher specificity for glucose (Howard and Sage,1969). Their effectiveness as glycoprotein stains was tested using known standard proteins. Gels were soaked in lectin-containing buffer (7.3.3). destained by diffusion, and photographed or scanned in ultraviolet light. Figure 3.7 shows gels of a glycoprotein and two non-glycoproteins, stained with Con A in the absence and in the presence of a monomeric ligand acting as a competitor to the glycoprotein. Horseradish peroxidase

(Figure 3.7 (A) containing 20% carbohydrate (w/w)(Handbk. Biochem.) binds Con A strongly. Total inhibition of binding in the presence of 0,1M α-D methyl mannoside indicates a high degree of binding specificity. Cytochrome c (Figure 3.7 (B)), a non glycoprotein, shows no binding of either LCH or Con A in the absence or presence of 0,1M ligand. However, strongly basic proteins like lysozyme or histones bind both lectins even in the presence of inhibiting sugar. Therefore the inhibition of lectin binding by a competing ligand such as α-D-methyl mannoside can be taken as proving the presence of a recognizable glycosidic chain in a glycoprotein.

The broad specificity of Con A for sugars is indicated by the high uptake of Fluorescein-Con A, whereas LCH binds to a lesser degree. With the given specific activity of the fluorescein labelled lectins, 100 ng of protein bound carbohydrate was easily detectable and nonspecific binding was reliably monitored by staining in the presence of ligand. Due to the broader specificity of Con A, this lectin was used as a routine glycoprotein stain in gels. Con A which recognizes mannose, is also a good choice as a stain for nuclear envelope glycoproteins as the nuclear envelope is particularly rich in mannose (Franke et al.,1976). A similar procedure has been developed in another laboratory (Virtanen,1977).

Detection of glycoproteins by the silver staining technique of Dubray and Bezard (1982) is based on the generation of aldehydic groups by oxidation of the 1,2 diol groups of carbohydrates with periodic acid. These groups then react with ammoniacal silver nitrate and the complex is visualized with formaldehyde developer (7.3). This method detects up to 5 ng of protein bound carbohydrate and is thus over 100 fold more sensitive then the Schiff-Periodate method. The method may also be adapted for staining proteins by replacing the periodate step with one which utilizes glutaraldehyde to cross link the proteins and provide free aldehyde groups for reaction with ammoniacal silver nitrate (7.3). While this stain is about 100 times more sensitive than Coomassie Brilliant Blue, for unknown reasons certain proteins which stain with Coomassie, have failed to show any staining with silver stain (Morrissey, 1981). The silver stain for glycoproteins has also been shown to bind the non glycoproteins, phosphorylase b and carbonic anhydrase, although with low sensitivity (Dubray and Bezard, 1982). The authors suggest that these proteins may in fact carry a few carbohydrate residues. The silver stain for glycoproteins was therefore used whenever possible in conjunction with lectin staining.

Periodate silver staining of standards is shown in Figure 3.7. The stain is highly sensitive and carbohydrate specific for the standards used. 100 ng of horseradish peroxidase is easily detected (Figure 3.7, A, g). This represents 20 ng of protein bound carbohydrate. 1 µg of either cytochrome c or lysozyme were not stained by this procedure (Figure 3.7 B, C). However, 5 µg of cytochrome c showed slight staining.

The bulk of the nuclear envelope proteins appear to be glycoproteins. Figure 3.8 (b) shows a gel of total nuclear envelope stained for glycoprotein with Fluorescein Con A. The lectin binds specifically to a large number of bands. Notable exceptions are those of a low molecular weight (±20 000 daltons). Prominently stained bands are the triplet at 60 000 daltons and the high molecular weight band (170 000 daltons). No binding is found when staining is done in the presence of α-D-methyl-mannoside, indicating that binding is due to specific interaction with a carbohydrate moeity rather than nonspecific adsorption.

Silver staining of nuclear envelope for protein and glycoprotein confirms the presence of a large number of glycoproteins (Figure 3.8). Prominently stained bands are again the 170 kilodalton and 60 kilodalton triplet bands. Most other bands stain to varying degrees. Gels stained with silver stain were 0,8 mm thick and had an acrylamide:bisacrylamide ratio of 60 : 1,6 rather than 60 : 0,4 in order to increase the rigidity of the gel which had to be handled frequently during staining and washing. The polypeptide pattern obtained on these gels thus differs from that on gels with a smaller percentage of bisacrylamide. The higher concentration of bisacrylamide, in general gave better definition of lower molecular weight bands, while gels with the lower bisacrylamide concentration allowed better separation of higher molecular weight polypeptides. Unless otherwise indicated in legends to figures, gels have been run using an acrylamide:bisacrylamide ratio of 60:0,4.

Attempts to visualize glycoproteins in gels using P.A.S. stain were less successful (Figure 3.8d). This stain, based on the generation of aldehydic functions by periodate oxidation, sensitive than the Con A stain. Excessively large amounts of nuclear envelopes were therefore needed before the presence of any glycoproteins could be shown. This resulted in gels which were grossly overloaded with respect to protein leading to poor resolution

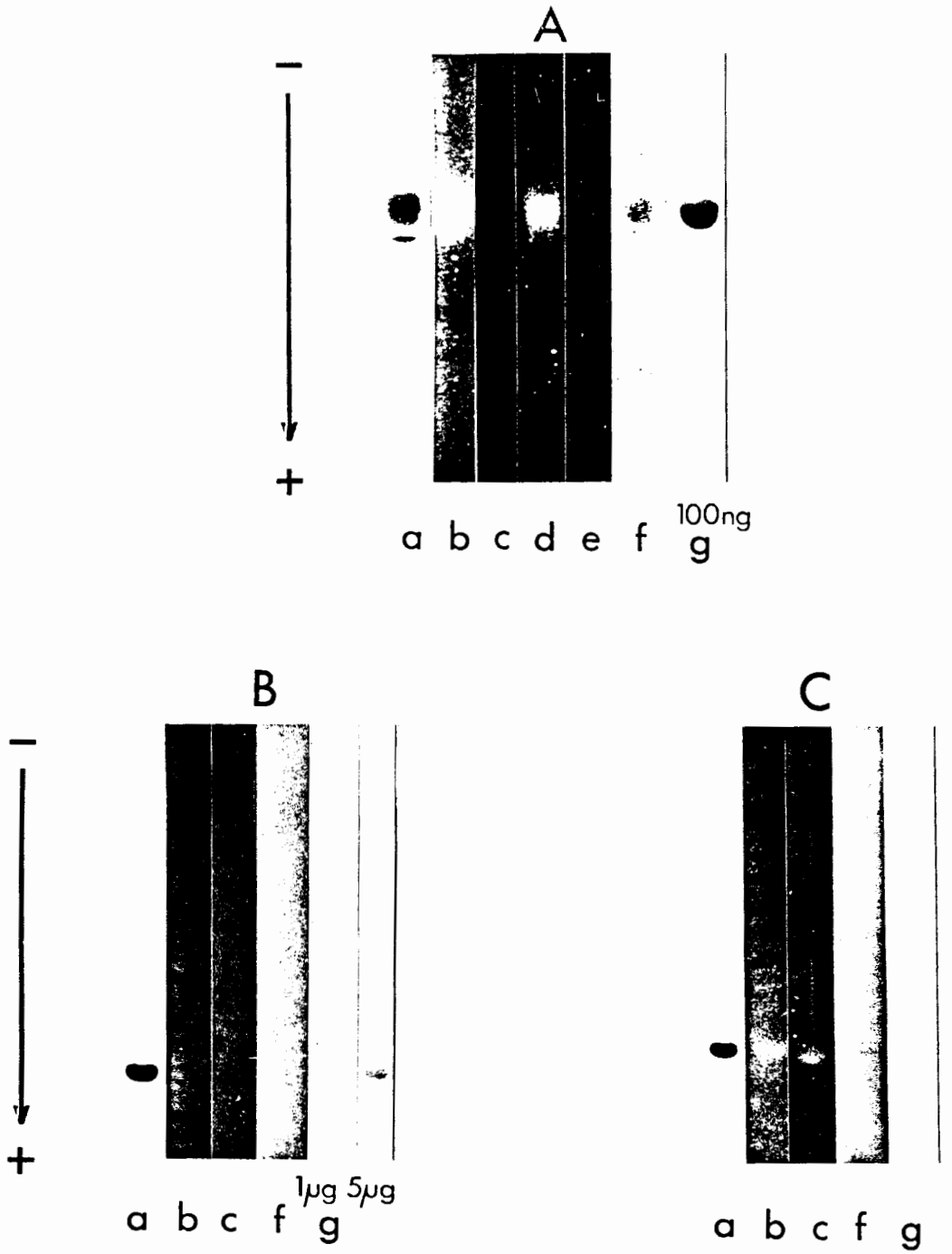

FIGURE 3.7 SDS gel electrophoresis of (A) Horseradish peroxidase (B) Cytochrome c (C) Lysozyme in 10% polyacrylamide gels for 3,5 hours at 100 V. 1 µg of protein was applied unless otherwise indicated.

(a) Stained with Coomassie Brilliant Blue
(b) Stained with Fluorescein Con A
(c) Stained with Fluorescein Con A in 0,1 M α-D-methyl-mannoside
(d) Stained with Fluorescein LCH
(e) Stained with Fluorescein LCH in 0,1 M glucose
(f) Stained with P.A.S.
(g) Stained with Silver after periodate oxidation

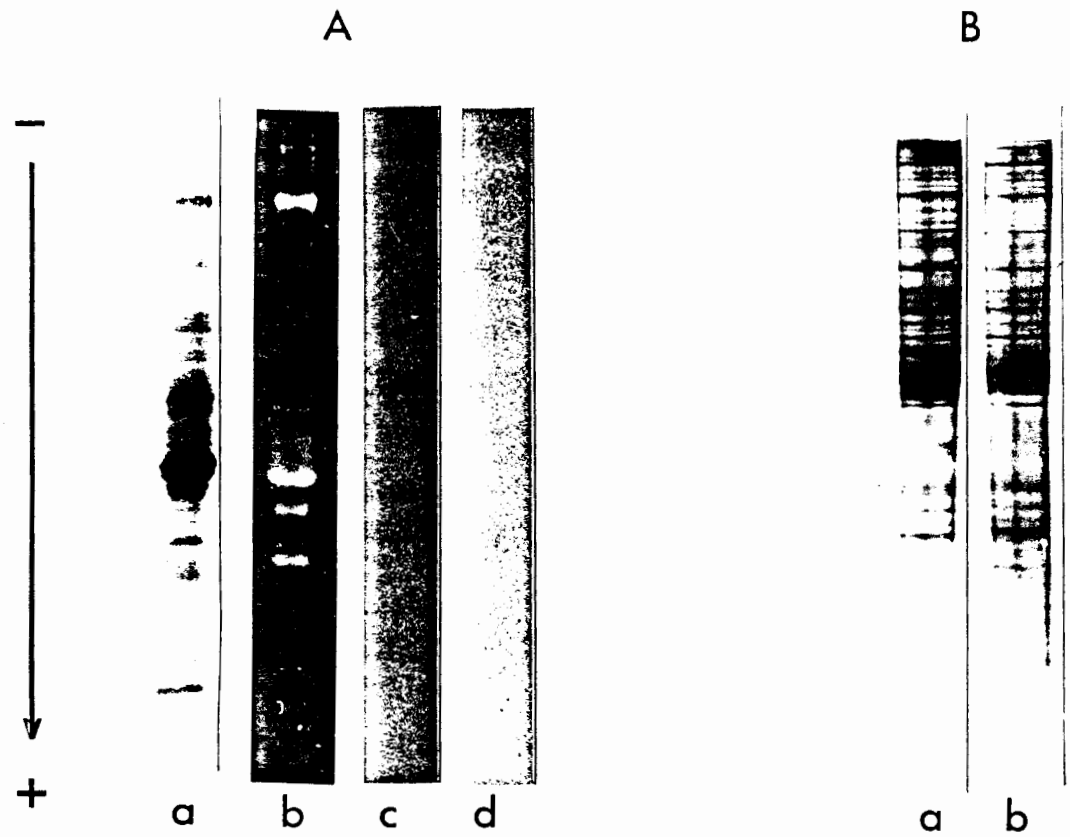

FIGURE 3.8

A. SDS gel electrophoresis of nuclear envelope for 3,5 hours at 100 V in a 10% polyacrylamide slab gel.

(a) Stained with Coomassie Brilliant Blue
(b) Stained with Fluorescein Con A
(c) Stained with Fluorescein Con A in a α-D methyl mannoside
(d) Stained with P.A.S.

B. SDS gel electrophoresis in a 10% polyacrylamide gel with an acrylamide:bisacrylamide ratio of 60:1,6 for 3,5 hours at 150V.

(a) Stained with silver for protein
(b) Stained with silver for glycoprotein

of individual bands. Even with gross overloading, bands were only faintly detectable using P.A.S. This may explain why there have been so few earlier reports on the glycoprotein nature of nuclear envelope proteins.

The presence of glycoproteins in the nuclear envelope has been confirmed by a number of authors (Kawasaki and Yamashina, 1972; Virtanen and Wartiovaara, 1976; Virtanen, 1977; Monneron and Segratain, 1974). Virtanen and Wartiovaara (1976) have found that Con A binds specifically to the cisternal faces of the inner and outer membranes of the envelope, presumably due to the carbohydrate moeities of glycoproteins as glycolipids have not been detected in the nuclear envelope.

3.3. CHROMATIN

3.3.1 Isolation

A large number of methods are available for the isolation of chromatin from purified nuclei. Virtually any procedure which disrupts nuclei gives rise to an insoluble fraction which may be referred to as chromatin. Chromatin may thus potentially contain all insoluble nuclear components. The method chosen here for chromatin isolation is that of Bonner et al. (1968) and is described in detail in Methods (7.2.3). Nuclei were disrupted in 0,05 M Tris, pH 8,0 and insoluble material pelleted and washed twice in the same buffer. The final pellet was sedimented through 1,7 M sucrose in order to remove membrane fragments which sediment at the 0,05 M Tris/1,7 M sucrose interface. The final pellet was designated chromatin.

3.3.2 Characterization: Isolation of Nonhistone Proteins from Chromatin

In order to establish whether nuclear envelope components appear as contaminants in chromatin despite sedimentation through 1,7 M sucrose, the nonhistone fraction was investigated. Nonhistone proteins were isolated from chromatin (7.2.4), subjected to SDS gel electrophoresis and stained either for protein with Coomassie Brilliant Blue or glycoprotein with fluorescein Con A.

Comparison of Coomassie stained gels indicated little overall similarity between the polypeptide distribution of the nonhistone fraction and that of the nuclear envelope (Figure 3.9). However when gels were stained for carbohydrate, a similarity in the glycoprotein content of the two fractions was immediately evident (Figure 3.11 (A,C)).The distribution of Con A binding polypeptides in both fractions was almost identical, although there were proportionately fewer glycoproteins per total amount of protein present in the nonhistone fraction. This evidence strongly suggested that the nuclear envelope constitutes a major chromatin contaminant. Some authors (Goldberg et al., 1978; Sevaljevic et al., 1979; Stein et al., 1975) have suggested that besides their presence in the nuclear envelope, glycoproteins are also bona fide components of eukaryotic chromatin. The results presented here indicate that the bulk of the Con A positive chromatin glycoproteins, from their overall distribution after SDS gel electrophoresis, appear to derive from the envelope. An attempt was therefore made to isolate nuclear envelope fragments from chromatin in order to establish whether these would account for the presence of glycoproteins in chromatin.

3.3.3 Isolation of nuclear envelope fragments from Chromatin

Nuclear envelope fragments were isolated from chromatin by the method employed when nuclei were used (7.2.2). By a number of criteria the fragments isolated from chromatin by heparin treatment were identical to the nuclear envelope. On gradient centrifugation they had a density identical to that of the nuclear envelope (Figure 3.10). They were indistinguishable on SDS gel electrophoresis whether stained for protein or carbohydrate (Figure 3.11 (A,B)). The envelope fragments also had a protein:DNA ratio of 30:1 - identical to that found for the nuclear envelope. Membrane fragments isolated from chromatin clearly derive from the nuclear envelope and the envelope must therefore be considered as a significant chromatin contaminant even after attempts at its removal by centrifugation through 1,7M sucrose.

To ascertain whether all chromatin Con A-positive glycoproteins could be accounted for by the contaminating nuclear membrane fragments, histones and nonhistones were isolated from the soluble heparin supernatant fraction (7.2.5) run on SDS gels and stained with Coomassie

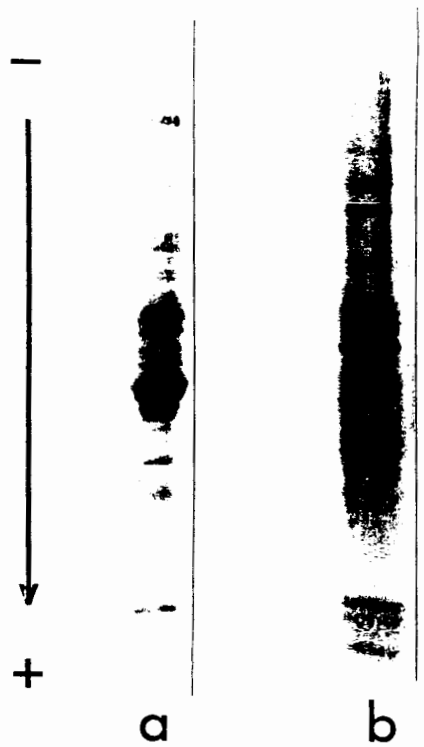

FIGURE 3.9 SDS gel electrophoresis of
(a) nuclear envelope
(b) total nonhistone proteins isolated as described in 7.2.4. Gels were stained with Coomassie Brilliant Blue.

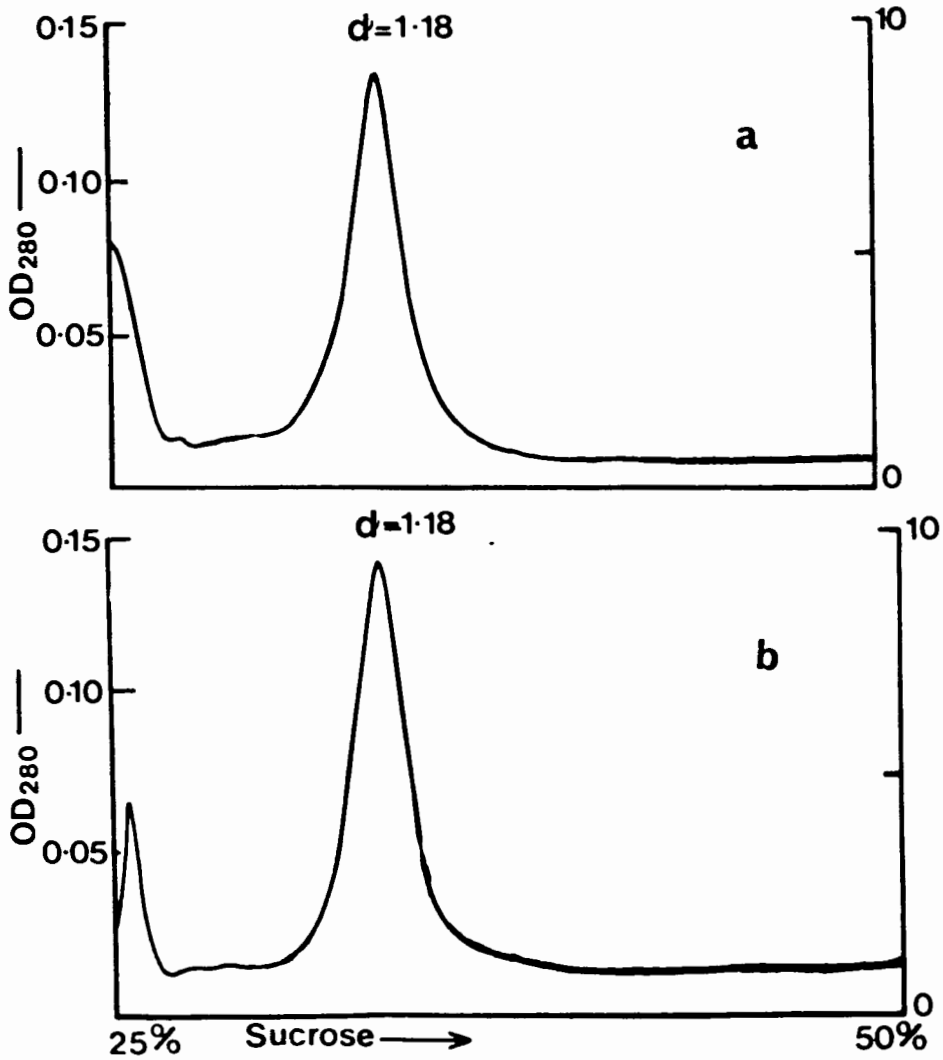

FIGURE 3.10 Sedimentation diagrams of (a) nuclear envelope and (b) nuclear envelope fragments isolated from chromatin centrifuged on a 25 - 50% sucrose gradient for 3 hours at 170 000g.

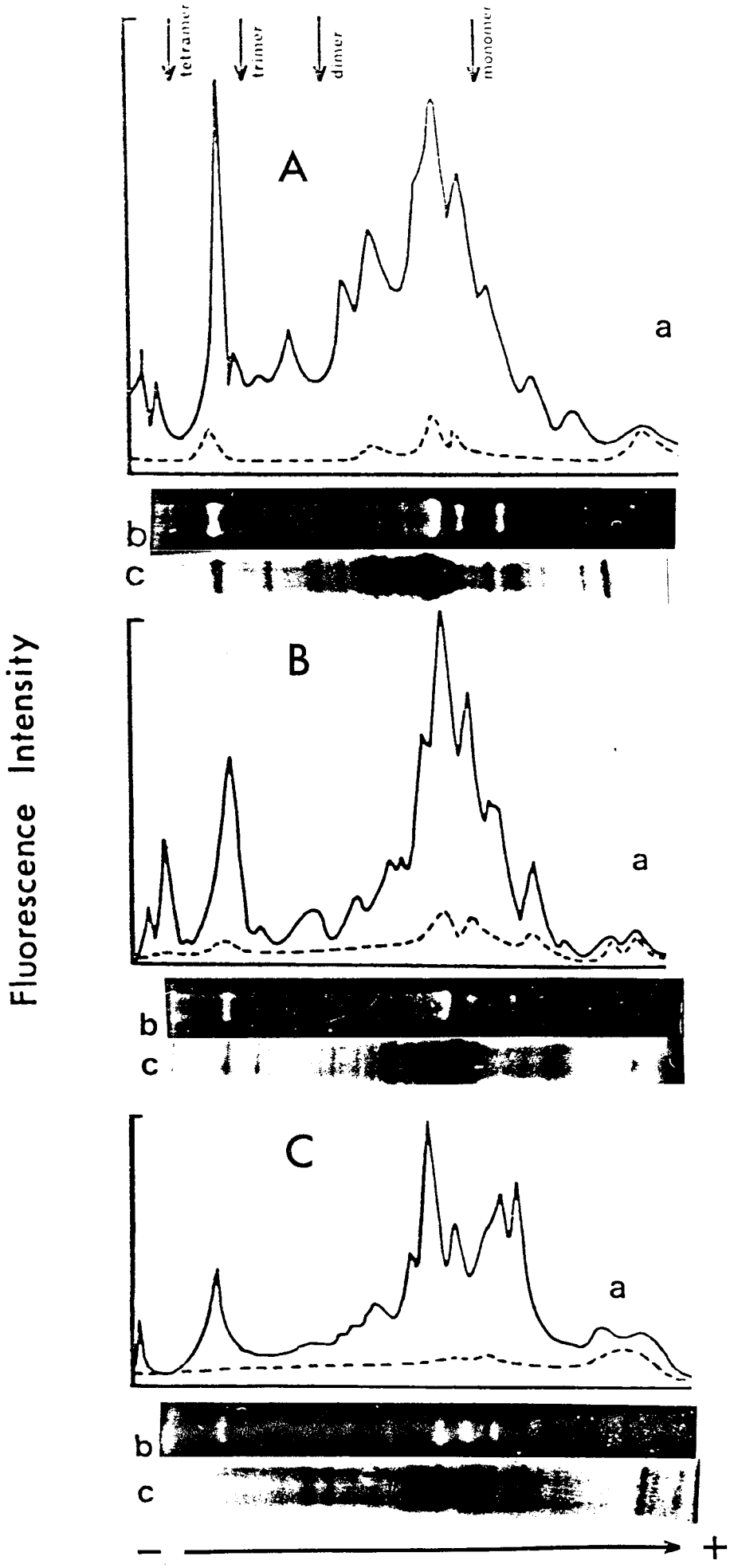

FIGURE 3.11 For legend, see following page.

FIGURE 3.11 (legend)

SDS gel electrophoresis of nuclear envelope (A), nuclear envelope fragments (B) and non-histone proteins (C). Gels were stained for protein with Coomassie Brilliant Blue and glycoproteins were visualized using Fluorescein labelled Con A. Nonspecific binding of Con A was monitored by staining in the presence of 0,1M α-D-methyl mannoside. The monomer of the molecular weight marker protein has a molecular weight of 53 000 Daltons (BDH product No. 44230).

a) Scans of the fluorescent gels
(————————) binding in the absence of α-D-methyl mannoside
(---------) binding in the presence of 0,1M α-D-methyl mannoside

b) Photograph of fluorescent gels

c) Protein stain of gels

Brilliant Blue and Con A. The results are shown in Figure 3.12. The soluble nonhistone fraction of chromatin has an entirely different polypeptide distribution from that of the nuclear envelope and is notably deficient in bands in the 50 - 60 kilodalton and 170 kilodalton range, which are major envelope components. The presence of glycoproteins in this fraction was not detectable with fluorescein Con A (Figure 3.12 (B)).

These results suggest that a distinct fraction of nonhistone chromosomal proteins from conventionally isolated chromatin belong to the nuclear envelope and that mannose rich chromatin glycoproteins in particular are of envelope origin. This finding is in keeping with the evidence that most nuclear carbohydrate may be accounted for by the nuclear envelope(Franke et al., 1976). Specific Con A binding may thus well be a good marker for nuclear envelope contamination of chromatin, bearing in mind that Con A also binds nonspecifically to the basic histones. A number of reports have noted specific lectin binding to chromatin and have suggested that glycoproteins may be specific genome-linked components (Sevaljevic et al., 1979; Goldberg et al., 1978). The results presented above suggest that the reported presence of glycoproteins in chromatin may be due to persistent envelope contamination.

3.4 ISOLATION OF OTHER MEMBRANES

3.4.1 Isolation of plasma membranes

The isolation of plasma membranes is described in 7.2.6. By the method employed, two batches of plasma membrane could be obtained from a single isolation; the first from the supernatant fraction after nuclei had been pelleted by low speed centrifugation, and the second from the crude nuclear pellet. In order to obtain minimum contamination by nuclear envelopes, only the membranes from the supernatant fraction were used. The plasma membrane banded at d = 1,17 in a 25 - 50% sucrose gradient (Figure 3.13).

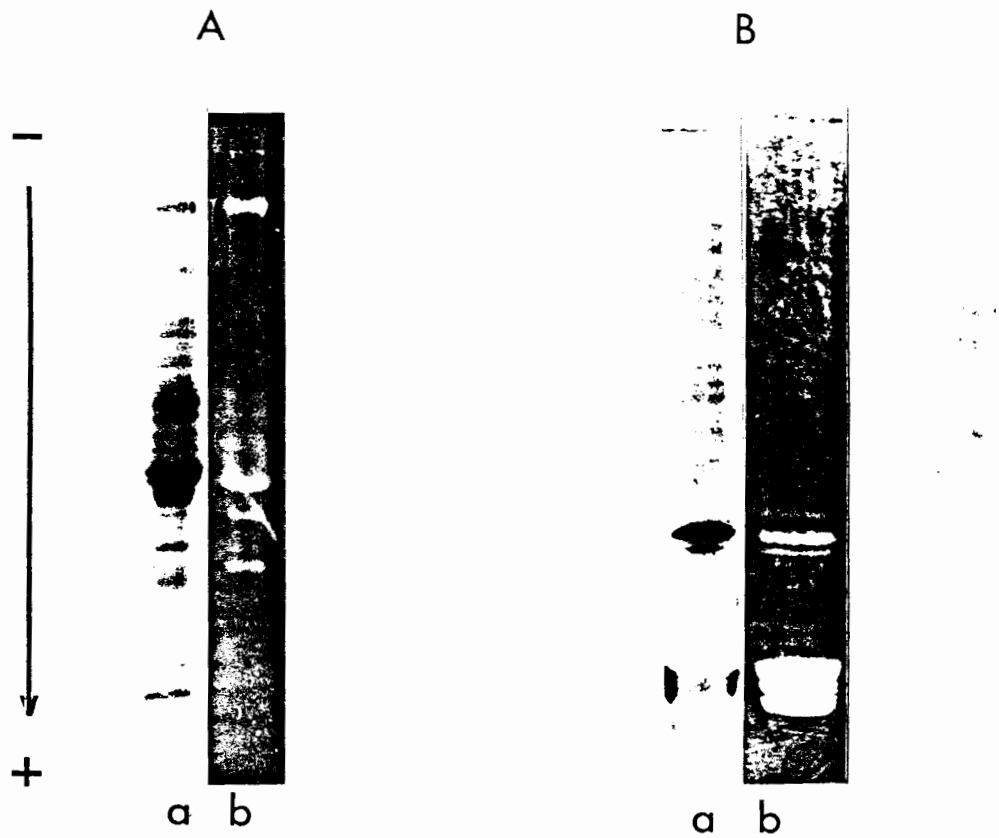

FIGURE 3.12 SDS gel electrophoresis of (A) nuclear envelope (B) heparinate supernatant fraction after removal of nuclear envelopes by centrifugation.

(a) Stained with Coomassie Brilliant Blue

(b) Stained with Fluorescein Con A

3.4.2 Isolation of microsomal membranes

Rough and smooth endoplasmic reticulum membranes were isolated as described in 7.2.7. Each membrane exhibited a characteristic density on a 25 - 50% sucrose gradient (Figure 3.13). Both rough and smooth endoplasmic reticulum have a higher density than the nuclear envelope and band at d = 1,195 and 1,19 respectively.

3.4.3 Characterization of microsomal and plasma membranes

The membranes were analysed by SDS gel electrophoresis and the protein and glycoprotein distribution compared with that of the nuclear envelope (Figure 3.14).

The polypeptide pattern produced on an SDS gel stained for protein showed a degree of similarity between the polypeptide composition of the various membranes (Figure 3.14A). This is particularly true for the approximately 55 000 dalton triplet present in all four membrane types and the lower molecular weight polypeptides. Smooth and rough endoplasmic reticulum have the 170 000 dalton band prominent in the nuclear envelope although this is absent in the plasma membrane. The plasma membrane does however possess a number of even higher molecular weight polypeptides. The similarity in polypeptide composition between the endoplasmic reticulum and the nuclear envelope was not unexpected in view of the proposed "in vivo" continuity of the endoplasmic reticulum with the outer nuclear membrane based on electron microscopic evidence. This similarity as well as similarities in the enzyme profile and lipid content of the two membranes has been noted by a number of authors (Harris, 1978; Franke et al., 1976; Kasper, 1974; Zbarsky, 1978). The membranes were therefore assayed for DNA to check for possible contamination by the nuclear envelope. The results are expressed in Table 3.2. Only the nuclear envelope shows any significant DNA association. The values obtained for the other membranes represent the lower limit of sensitivity of the assay. Using larger amounts of membrane resulted in significant interference by the carbohydrate moeities of the membrane glycoproteins.

TABLE 3.2

DNA CONTENT OF ISOLATED MEMBRANES

	µ g DNA/100µg PROTEIN
Nuclear envelope	5,10 ± 2,50
Rough endoplasmic reticulum	0,53 ± 0,50
Smooth endoplasmic reticulum	1,25 ± 0,25
Plasma membrane	1,15 ± 0,63

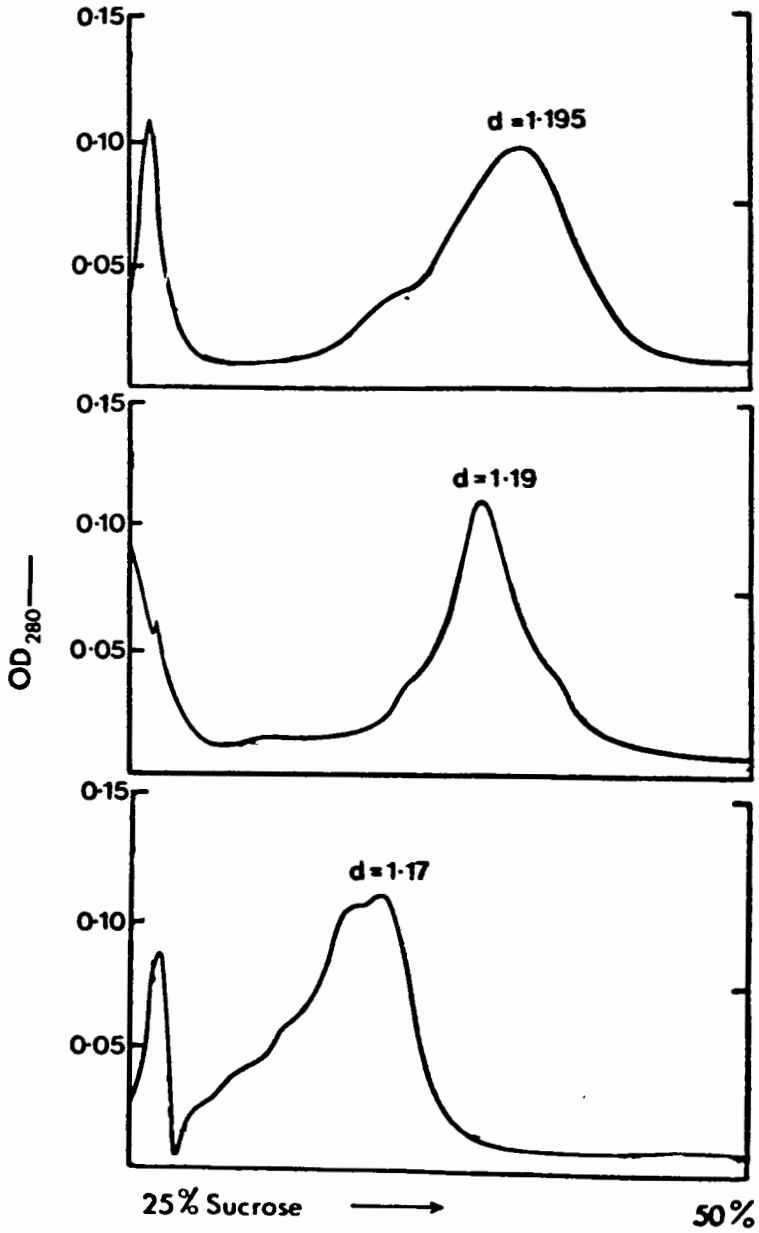

FIGURE 3.13

Sedimentation diagrams of microsomal and plasma membranes in a 25 to 50% sucrose gradient. The amount of membrane in each incubation was identical with respect to protein. Centrifugation was for 3,5 hours at 170 000g.

(a) Rough endoplasmic reticulum
(b) Smooth endoplasmic reticulum
(c) Plasma membrane

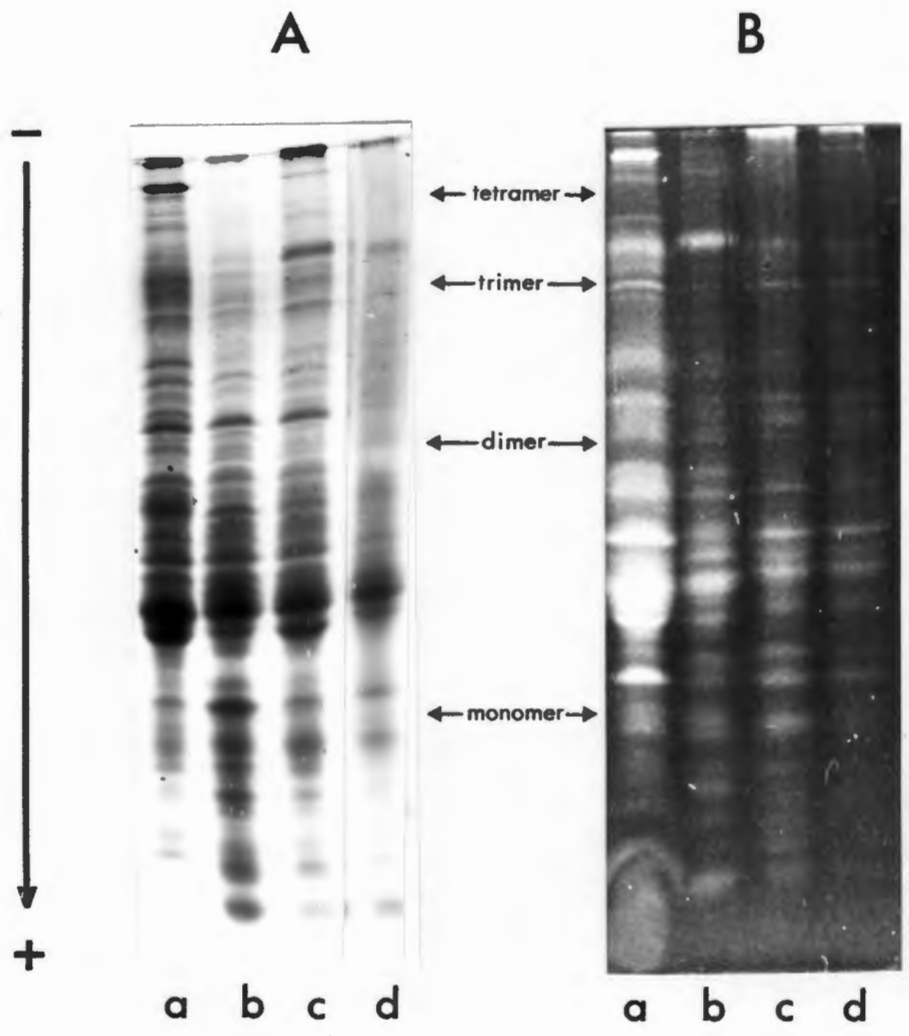

FIGURE 3.14

SDS gel electrophoresis of (a) plasma membrane (b) smooth endoplasmic reticulum (c) rough endoplasmic reticulum (d) nuclear envelope

A) Stained with Coomassie Brilliant Blue

B) Stained with Fluorescein Con A

Arrows indicate molecular weight marker proteins: Monomer = 53 000 daltons (BDH Product No. 44230)

The glycoprotein pattern as revealed by Con A staining presents a striking similarity in the distribution of glycoproteins in the membrane types (Figure 3.14). The nuclear envelope and two endoplasmic membranes have a near identical glycoprotein distribution, although there are quantitative differences in certain bands. The plasma membrane differs significantly from the other membrane types. These results confirm the reported similarity in carbohydrate content of the nuclear envelope and endoplasmic membranes (Franke et al., 1976; Bornens and Kasper, (1973).

3.5 DISCUSSION

The nuclear envelope prepared by the heparin method comprises a two leaflet structure (Bornens, 1977) representing the outer and inner membrane of the nucleus. Although electron micrsocope techniques were not available during this study, the envelope preparation had a composition and a density similar to that found by other authors for the nuclear envelope.

The non-histone protein fraction of chromatin is most frequently cited as containing the nuclear acceptor sites for steroid-hormone receptors (for review, O'Malley et al., 1977). However, in contrast to the nuclear envelope which is a reasonably well defined subfraction of the nucleus (Harris, 1978; Franke et al., 1976), the non-histone fraction of chromatin may be expected to vary considerably depending on the method chosen for either the chromatin or the subsequent non-histone isolation. Most authors include a density gradient centrifugation step in the preparation procedure for chromatin which serves as a starting material for the isolation of non-histones. This step is designed to remove the nuclear envelope. Results presented here show however that centrifugation through 1,7M sucrose used to isolate the chromatin preparation leaves a considerable amount of nuclear envelope fragments in the chromatin. Also a nonionic detergent such as Triton X-100 has been used by other to remove the outer nuclear membrane. As the outer nuclear membrane is no longer visible by electron microscopy after such a Triton treatment, and over 80% of phospholipid has been removed (Frederiks et al., 1978), the outer membrane is generally assumed to be solubilized in toto. However, it has been demonstrated that the nuclear pore complex remains associated with the nucleus after Triton X-100 treatment (Aaronson and Blobel, 1975) and that 1% Triton X-100 though removing virtually all of the phospho-

lipids removes only a small amount of nuclear envelope protein (Jackson, 1976). The single layer residual nuclear envelope with readily visible nuclear pores seen by electron microscopy (Berezney and Coffey, 1976) after Triton treatment of nuclei thus represents a collapsed bileaflet membrane and the assumption that Triton X-100 treatment removes the outer leaflet in toto is not justified.

Part of the Concanavalin A binding sites shown to be present in the nuclear membrane (Monneron and Segretain, 1974) are due to glycoproteins which are well established components of the nuclear envelope (for review, Franke et al., 1976). A number of authors (Stein et al., 1975; Rizzo and Bustin, 1977; Goldberg et al., 1978) have described the occurrence of glycoproteins in chromatin even after rigorous treatment with Triton. Though identity of mobility in SDS-gels only indicates similar molecular weights and the binding of Concanvalin A only proves the presence of a ligand within the limits of the specificity of the lectin, the striking similarity of the glycoprotein pattern revealed by the uptake of fluorescein-Concanavalin A after SDS-gel electrophoresis (Figure 3.11) for nuclear envelopes, nuclear envelope fragments isolated from purified chromatin and non-histones argues strongly that most of the glycoproteins in chromatin derive from envelope contamination.

Most comparative studies of endoplasmic membranes and the nuclear envelope have involved either an enzyme profile of the two membrane types or comparison of polypeptide composition on SDS polyacrylamide gels which show many similarities as well as a number of distinct differences, especially in the low molecular weight regions. The near identical glycoprotein distribution revealed by Con A staining was an unexpected finding. All the major middle and high molecular weight bands appear to be glycoproteins. Treatment of the envelope with 2M NaCl (Figure 3.15) did not extract any of the Con A stained bands, indicating that the bulk of glycoproteins are probably integral rather than peripheral proteins. Very little is know about the glycoprotein distribution or function in either membrane type. Recent work by Virtanen and co-workers (1976) showing the binding of Con A to only the cisternal surfaces of the nuclear envelope and the finding that the envelope contains a larger number of glycoproteins than suggested by earlier reports (Virtanen, 1977) indicates a possibly more prominent role for envelope carbohydrate than was previously thought.

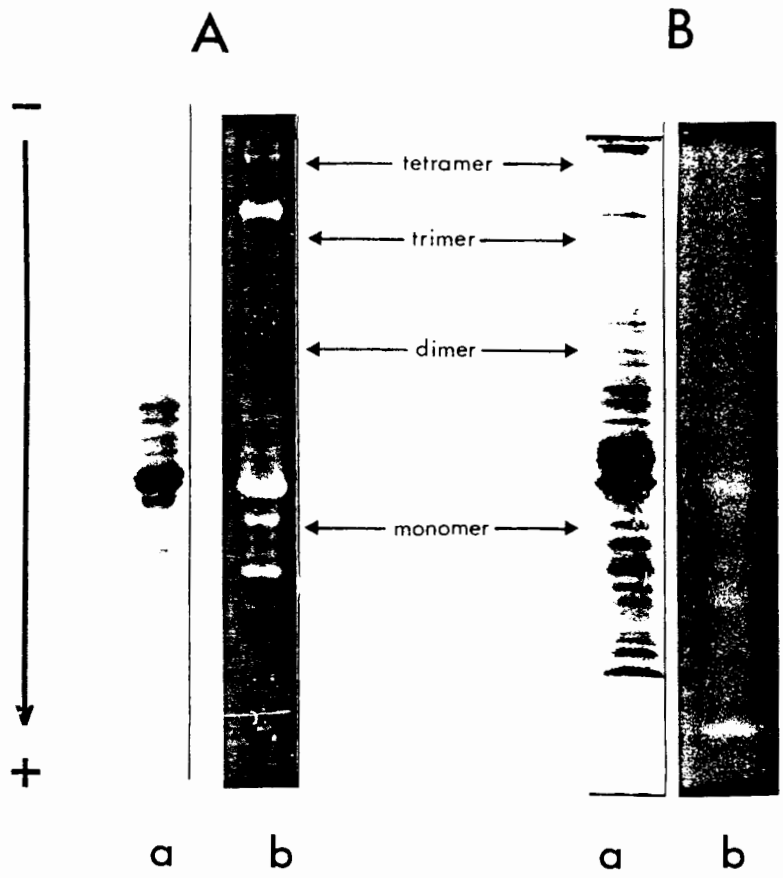

FIGURE 3.15 SDS gel electrophoresis of (A) nuclear envelope and (B) nuclear *envelope* extracted with 2 M NaCl

(a) Stained with Coomassie Brilliant Blue

(b) Stained with Fluorescein Con A

Arrows indicate molecular weight marker proteins: monomer = 53 000 daltons (BDH Product No. 44230).

PART 4

INTERACTION OF ACTIVATED TRIAMCINOLONE ACETONIDE-RECEPTOR COMPLEX WITH THE NUCLEUS AND SUBFRACTIONS OF THE NUCLEUS

Introduction

Differentiating between specific and nonspecific nuclear acceptor sites for cytoplasmic steroid hormone-receptor complexes in general and glucocorticoid receptor complexes in particular is a recurrent problem facing workers in the field. The complex binds nonspecifically to DNA at low ionic strengths. In binding studies of cytoplasmic glucocorticoid receptor to nuclear acceptor sites, nuclei or chromatin are in general pelleted by centrifugation after incubation with labelled steroid receptor and the pellet assayed for radioactivity. The resolubilization of the radioactively charged cytoplasmic steroid receptor-complex from the insoluble cytoplasmic steroid hormone receptor-nuclear acceptor complex in the presence of 0,3 - 0,4M KCl has been widely accepted as being indicative of the presence of specific binding sites and forms the basis of methods to determine such sites (Schrader et al.,1977). The interpretation of results from such tests is often difficult because the unbound receptor sometimes aggregates and pellets nonspecifically (Climent et al., 1977). In this study the binding of glucocorticoid hormone receptor complex to potential acceptor sites has, where possible, been investigated under conditions of sucrose gradient centrifugation at sucrose concentration ranges which keep the essential components of the test system well separated according to their buoyant densities.

4.1 INCUBATION OF TRIAMCINOLONE ACETONIDE RECEPTOR COMPLEX WITH NUCLEI AND CHROMATIN

The assay conditions used in the incubation of nuclei and chromatin with the Triamcinolone acetonide-receptor are fully described in Methods 7.4.2. Bound radioactivity was detected after incubation by extracting pelleted nuclei or chromatin with 0,3 M KCl. Both nuclei and chromatin bound the TA-receptor complex. In the concentration range tested, binding to both nuclei and chromatin was not found to be saturable. Increasing concentrations of

TA-receptor complex were incubated with a constant amount of either nuclei or chromatin. Results are expressed in Figure 4.1. These agree with results previously reported by others (Milgrom and Atger, 1975), and are in accordance with the well established affinity of the activated glucocorticoid receptor for DNA or other polyanionic matrices which forms the basis of a number of purification methods for the receptor (Climent et al., 1977; Wrange et al., 1979).

4.2 INCUBATION OF NUCLEAR ENVELOPE WITH TA-RECEPTOR COMPLEX

Nuclear envelope was incubated with TA-receptor complex and the reaction mixture analysed on a 25 - 50% sucrose gradient (see Methods 7.4.1). Results are represented in Figure 4.2(a). Radioactivity was localized in three places on the gradient. About 50% comigrated with the nuclear envelope. 40% remained at the top of the gradient. It was not possible to mop up this unbound radioactivity by increasing the amount of nuclear envelope (Figure 4.2(b)) confirming the finding (Section 2.2) that a percentage of the preparation was inactive. A small pellet representing about 10% of the radioactivity was usually obtained, representing probably aggregated TA-receptor. When centrifuged without envelope, the TA-receptor remained at the top of the gradient, and a small amount of aggregated material pelleted. Free triamcinolone acetonide incubated with the nuclear envelope failed to bind the envelope and remained at the top of the gradient after centrifugation (Figure 4.2(c)). The association of steroid with the envelope thus appears to be a protein mediated process.

Repeated freezing and thawing inactivated the TA-receptor complex which could then no longer be bound by the envelope. This inactivation was not due to dissociation of the steroid from its receptor, as the complex still chromatographed in the outer volume on a Sephadex G25 column. Incubation of the TA-receptor complex with nuclear envelope at $20^{o}C$ resulted in a considerable reduction in the amount of complex bound. All incubations and centrifugations were therefore carried out at $4^{o}C$. Other workers have found that the partially purified receptor complex is temperature labile (Wrange, 1976).

These results indicate that the binding of the TA-receptor complex to the

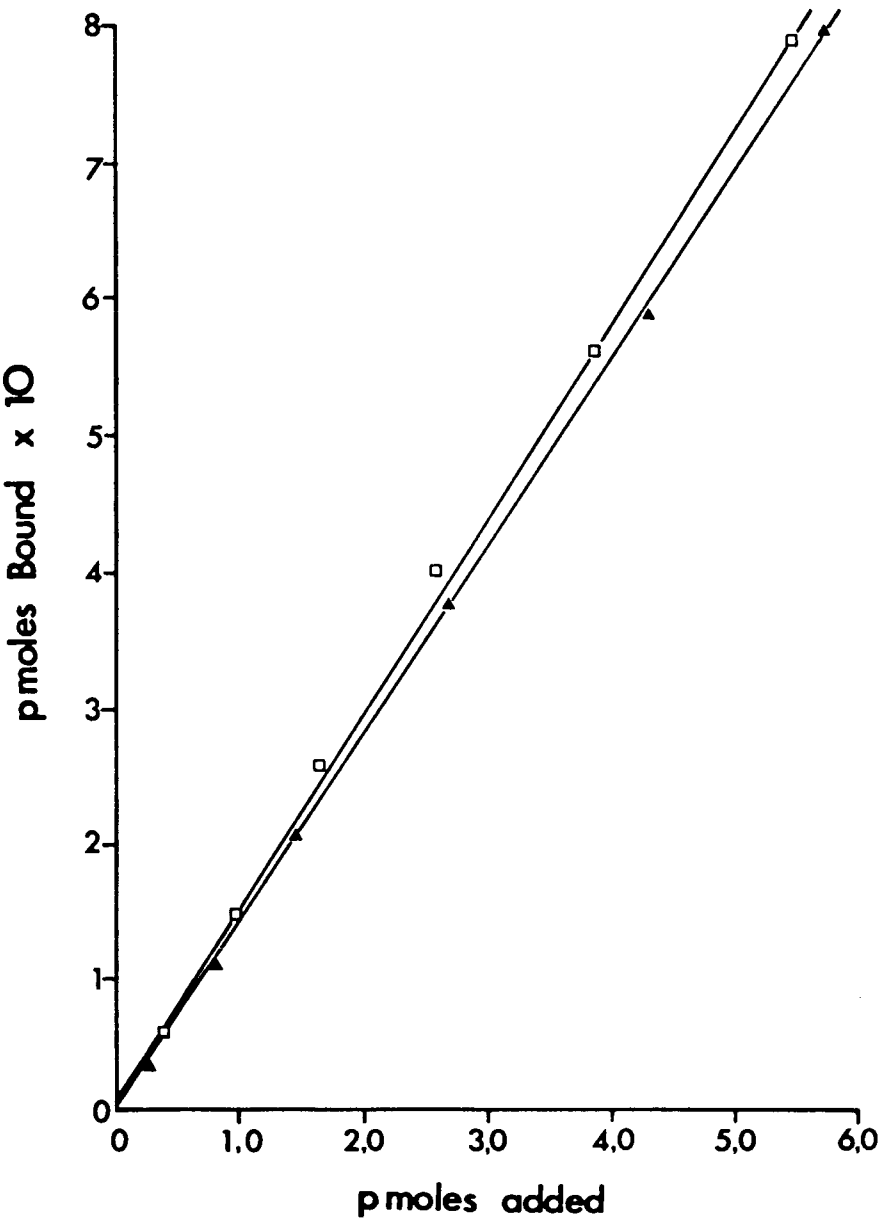

FIGURE 4.1 Saturation plot of TA-receptor complex binding to a constant amount of nuclei or chromatin (1 mg DNA). Prior to incubation, aggregated material was removed from the TA-receptor complex by centrifugation. Incubation conditions are described in Materials and Methods 7.4.2. Aggregation of the TA-receptor complex during incubation was monitored by using blanks which did not contain nuclei or chromatin.

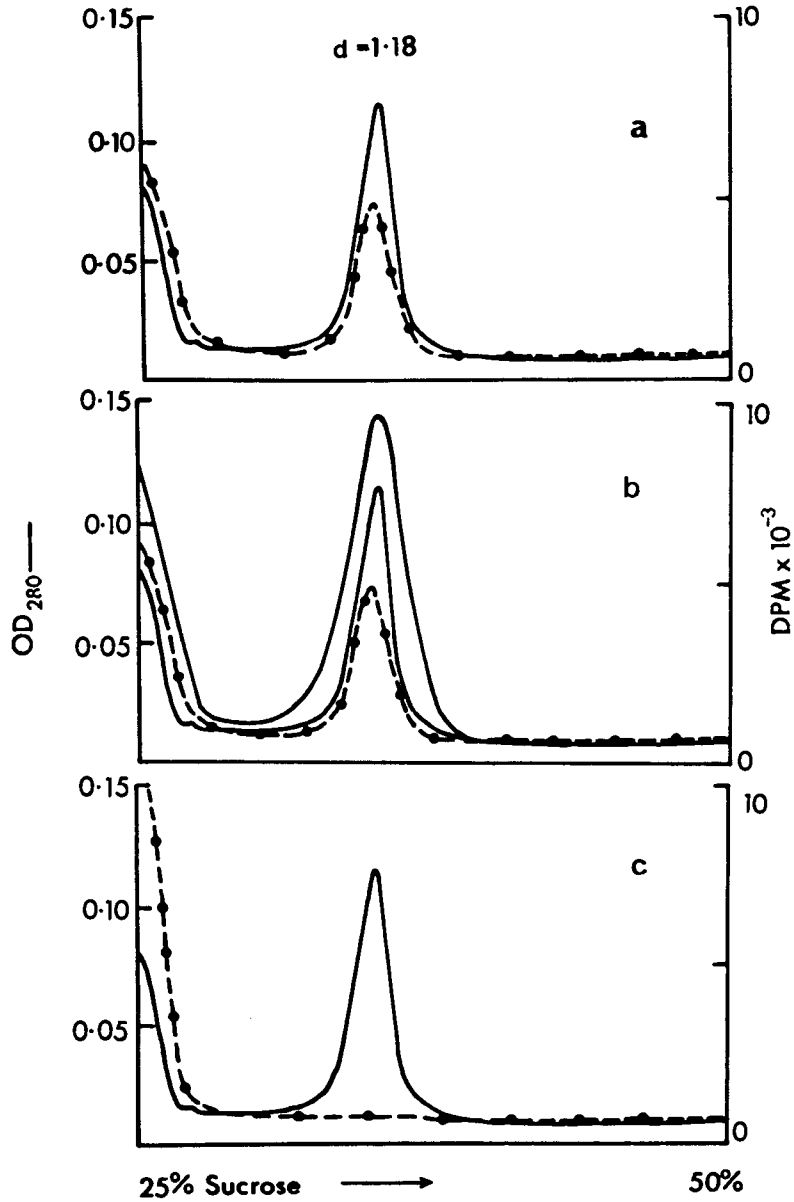

FIGURE 4.2 Sedimentation diagrams of nuclear envelope in a 25 - 50% sucrose gradient in TGA buffer after incubation with TA-receptor complex. Centrifugation was for 3,5 hours at 170 000g.

(a) Nuclear envelope incubated with receptor complex.

(b) Increasing amounts of nuclear envelope incubated with a constant amount of TA-receptor complex.

(c) Nuclear envelope incubated with free triamcinolone acetonide.

——— OD_{280}

----- DPM

nuclear envelope is a protein mediated event which is highly dependent on the activity of the complex. The fact that after repeated freezing and thawing or at elevated temperatures the complex no longer binds the envelope at all, is good evidence that binding is related to the activity of the complex and is not due to non-specific protein adsorption to the membrane.

To investigate the effect of ionic strength on binding, the ionic strength in the gradients and the incubation medium was increased to 0,15M and 0,3M KCl. At 0,15M KCl, no decrease in binding was observed (Figure 4.3(a)) but on further increase to 0,3M KCl, complete dissociation of radioactivity from the envelope was observed (Figure 4.3(b)) and the bulk of radioactivity was found at the top of the gradient. The extraction of steroid-hormone receptor complexes from nuclei and chromatin with 0,3M NaCl has been previously reported (Schrader et al., 1977). The TA-receptor complex can be similarly extracted from the nuclear envelope with 0,3M KCl. The envelope itself appeared to undergo a structural change in the presence of 0,3M KCl witnessed by the fact that it sedimented at a lower density d = 1,14 - 1,15 (Figure 4.3(b)).

To establish whether the binding of the TA-receptor to the nuclear envelope did take place in intact nuclei, nuclear envelopes were prepared from nuclei which had been preincubated with TA-receptor complex. Approximately 20% of the TA receptor which pelleted with the nuclei was recovered in the nuclear envelopes isolated from these nuclei (Figure 4.4, Table 4.1). The labelled receptor dissociated typically from the envelopes at elevated ionic strength (Figure 4.4). Although only 20% of nuclear bound TA receptor was recovered in the nuclear envelope, this value is significant in view of the presence of the large excess of polyanionic competitor in the form of DNA released during treatment of the nuclei with heparin. The binding of the TA-receptor to the nuclear envelope in intact nuclei may thus be masked by nonspecific binding to the large excess of polyanionic binding sites.

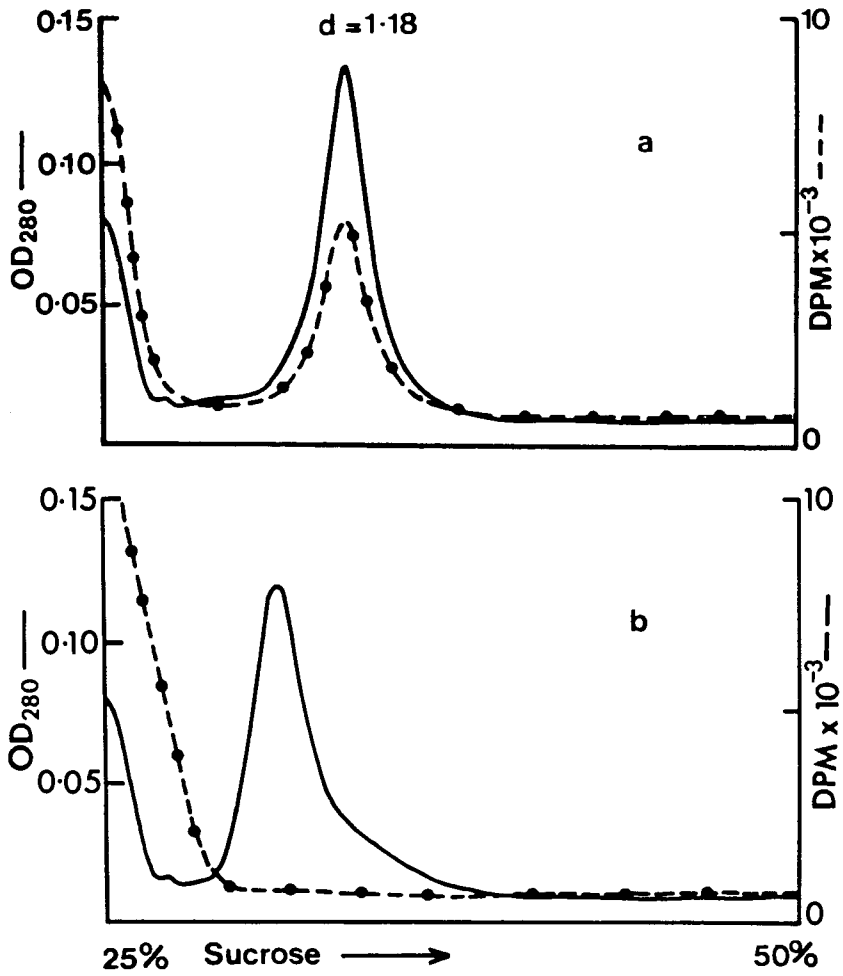

FIGURE 4.3 Sedimentation diagrams of nuclear envelope in a 25 - 50% sucrose gradient under conditions described in Figure 4.2.

(a) Nuclear envelope incubated with TA-receptor complex in TCA buffer. Both incubation medium and gradient were 0,15M KCl .

(b) Conditions as in (a) but incubation medium and gradient contain 0,3M KCl.

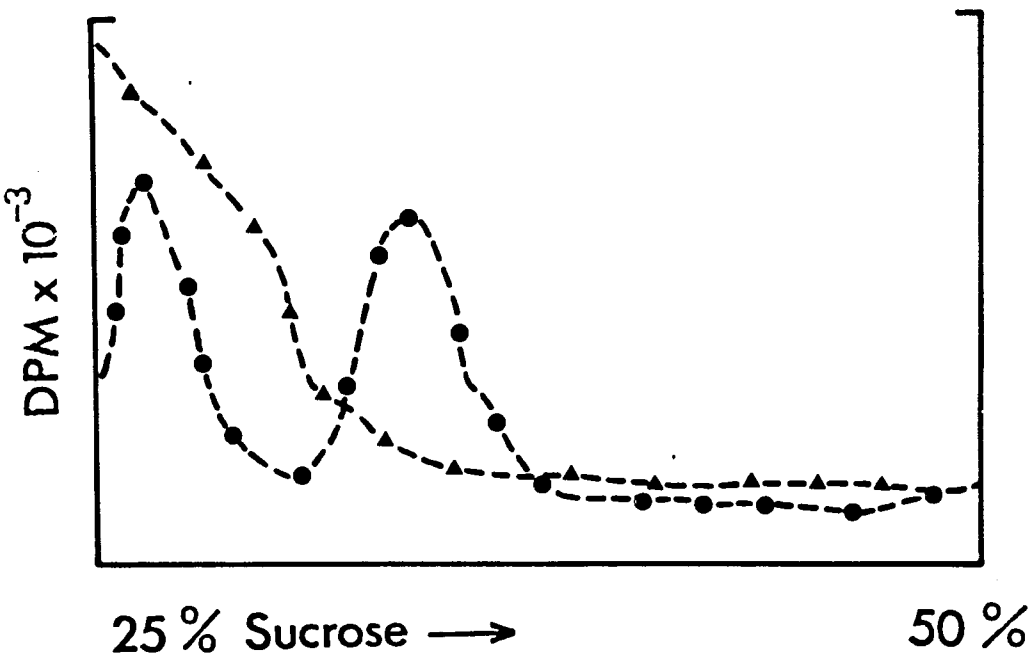

FIGURE 4.4 Sedimentation diagram of nuclear envelopes isolated from nuclei preincubated with TA-receptor complex (see Table 4.1).

- ● Sucrose gradient in 0,1M KCl
- ▲ Sucrose gradient in 0,3M KCl

TABLE 4.1

BINDING OF TA-RECEPTOR COMPLEX TO NUCLEAR ENVELOPES ISOLATED FROM LABELLED NUCLEI

INPUT RADIOACTIVITY dpm	NUCLEAR PELLET dpm	NUCLEAR ENVELOPE ISOLATED FROM LABELLED NUCLEI dpm
25 000	7 700	2 500

Nuclei were incubated with TA-receptor complex as described (7.4.1) and pelleted by centrifugation. Nuclear envelopes were isolated from pelleted nuclei as previously described (7.2.2).
Results represent an average of three determinations.

To further establish that the retention of radioactivity by the nuclear envelope after exposure to highly labelled TA-receptor complex was TA-receptor mediated, a competition experiment using "unlabelled" TA-receptor was performed. "Unlabelled" receptor complex was prepared by the same methods using triamcinolone acetonide with a specific activity one-tenth of that used for highly labelled complex (See 2.1).

The results of the competition experiments are expressed in Table 4.2. Nuclear envelopes were preincubated with a 6,6 and 9,0nM concentration of "unlabelled" TA-receptor for 60 minutes, pelleted, washed and reincubated with highly labelled TA-receptor. The "unlabelled" TA-receptor was successfully able to compete with the highly labelled complex for acceptor sites on the envelope.

TABLE 4.2

COMPETITION OF RADIOACTIVE AND NON-RADIOACTIVE CYTOPLASMIC TA-RECEPTOR COMPLEX FOR NUCLEAR ENVELOPE SITES

INPUT RADIOACTIVE CYTOPLASMIC TA-RECEPTOR (dpm)	TA-RECEPTOR BOUND TO ENVELOPE (dpm)		
	NO PRETREATMENT WITH COMPETITOR	PRETREATMENT WITH COMPETITOR	
		6,6n MOLAR	9 n MOLAR
2950	710	352	150
5670	1420	948	740
9212	3015	1503	1260

Nuclear envelopes were pre-incubated at 6.6 and 9 nmolar concentration of unlabelled TA receptor for 60 minutes, pelleted, washed and reincubated with radioactive TA-receptor.

4.3 INTERACTION OF TA-RECEPTOR COMPLEX WITH NUCLEAR MEMBRANE FRAGMENTS FROM CHROMATIN

Nuclear membrane fragments isolated from chromatin were incubated with TA-receptor complex under identical conditions to those employed for the nuclear envelope. These fragments bound the TA-receptor complex in a fashion identical to that of the nuclear envelope (Figure 4.5). This means that a proportion of the TA-receptor binding sites in chromatin can be accounted for by nuclear envelope fragments although these sites are probably masked by gross binding to nonspecific DNA sites.

4.4 SCATCHARD ANALYSIS

Unlike the nonsaturable binding characteristics of nuclei and chromatin, binding sites for the TA-receptor in the nuclear envelope and nuclear membrane fragments were found to be saturable. Increasing the amount of TA-receptor for a fixed amount of nuclear envelope led to increased binding until saturation was reached at 0.015pmole TA-receptor per µg of membrane protein (Figure 4.6(a)). Essentially identical binding was obtained for nuclear envelope and nuclear membrane fragments. The shape of the saturation curve indicated more than one binding site and Scatchard analysis (Figure 4.6(b)) revealed the presence of two high affinity sites in the nuclear envelope for cytoplasmic TA-receptor complex with formation constants of $4{,}5 \times 10^{-9}$ and 6×10^{-10} M. More than one nuclear acceptor site for cytoplasmic steroid hormone-receptor complex has been revealed by others for progesterone in oviduct cells (Spelsberg et al., 1976).

The similarity of the Scatchard plots obtained for nuclear envelope and nuclear membrane fragments provides further evidence that these membrane fragments originate from the nuclear envelope.

4.5 BINDING OF TA-RECEPTOR COMPLEX TO OTHER MEMBRANES

The isolation and characterization of rat liver plasma membrane and the rough and smooth endoplasmic membranes is described in 7.2.6 and 7.2.7. In order to establish whether binding of the TA-receptor complex to the nuclear envelope was specific for that membrane type, the binding of the complex to other membranes of the liver cell was investigated.

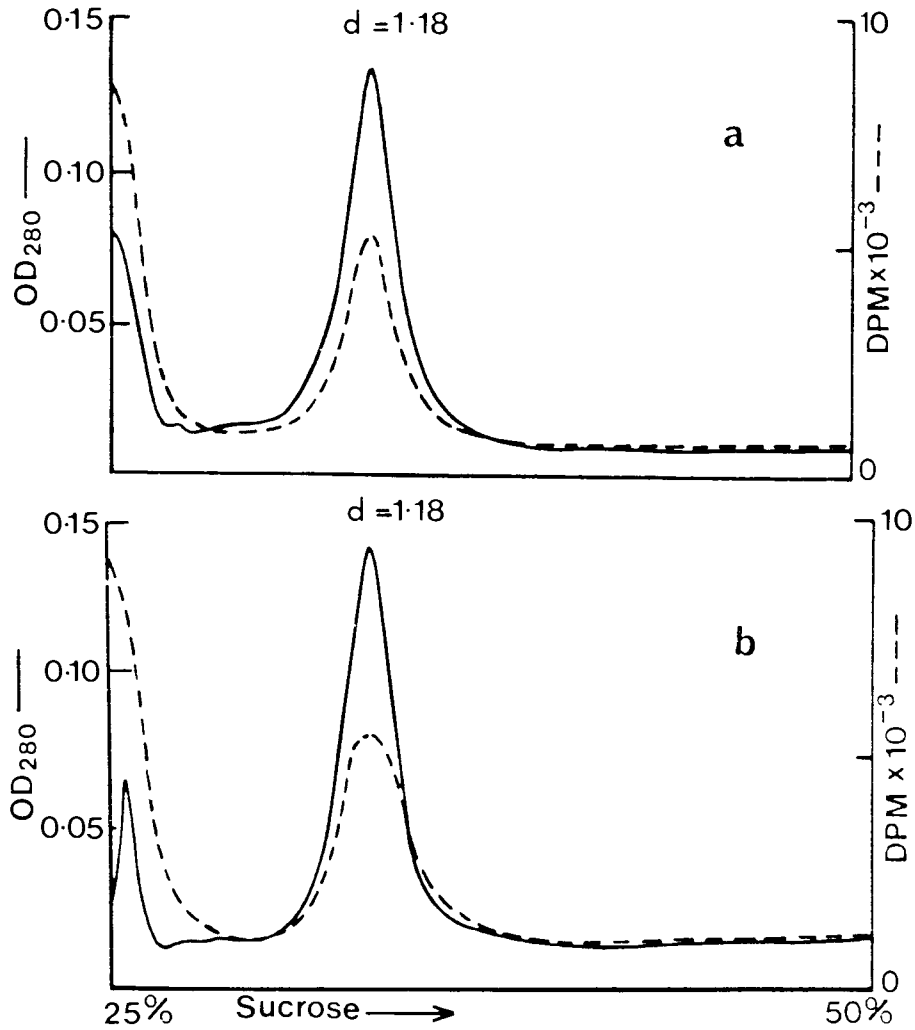

FIGURE 4.5 Sedimentation diagrams of nuclear envelope (a) and nuclear envelope fragments (b), the latter isolated from chromatin in a 25 - 50% sucrose gradient in TGA buffer after incubation with cytoplasmic steroid hormone receptor complex for 1 hour at +4°C. The amount of nuclear envelope and envelope fragments in each incubation was identical with respect to protein. Centrifugation was for 3.5 hours at 170 000g.

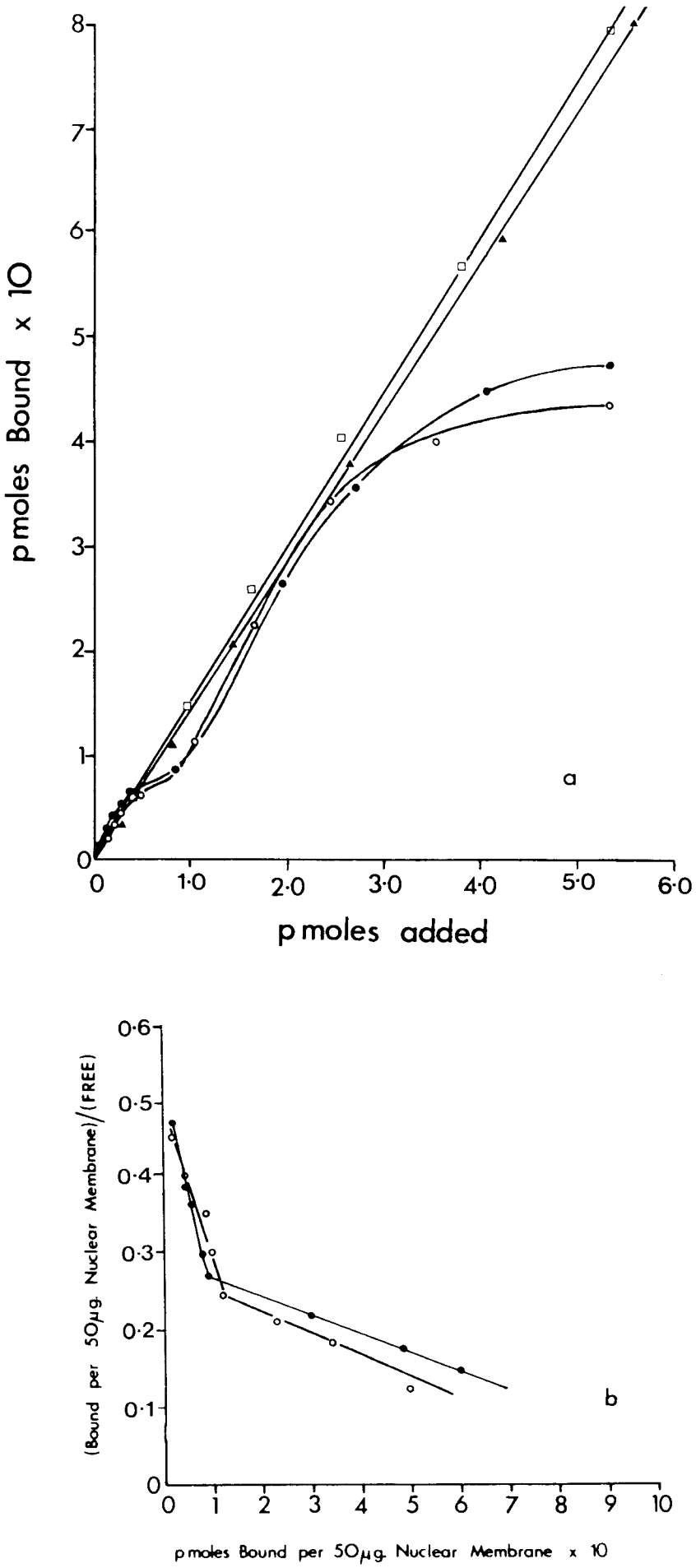

FIGURE 4.6 For legend, see next page.

FIGURE 4.6 (legend)

a) Saturation plot of hormone receptor binding to a constant amount of nuclear envelope. Prior to incubation with nuclear envelope, aggregated material was removed from the hormone receptor complex by centrifugation (100 000g for 1 hour). Incubation took place in 300 µl TGA buffer for 1 hour at +4°C after which the nuclear envelope hormone receptor complex was pelleted by centrifugation. The supernatant was carefully removed and the pellet washed with 100 µl 0.3M KCl which was assayed for radioactivity. Aggregation of the hormone receptor complex during the experiment was monitored by using blanks with appropriate cytoplasmic receptor concentrations which did not contain nuclear envelope. Non specific hormone receptor aggregation was found to be between 10% and 20%.

b) Scatchard analysis of the data in Figure 4.6(a).

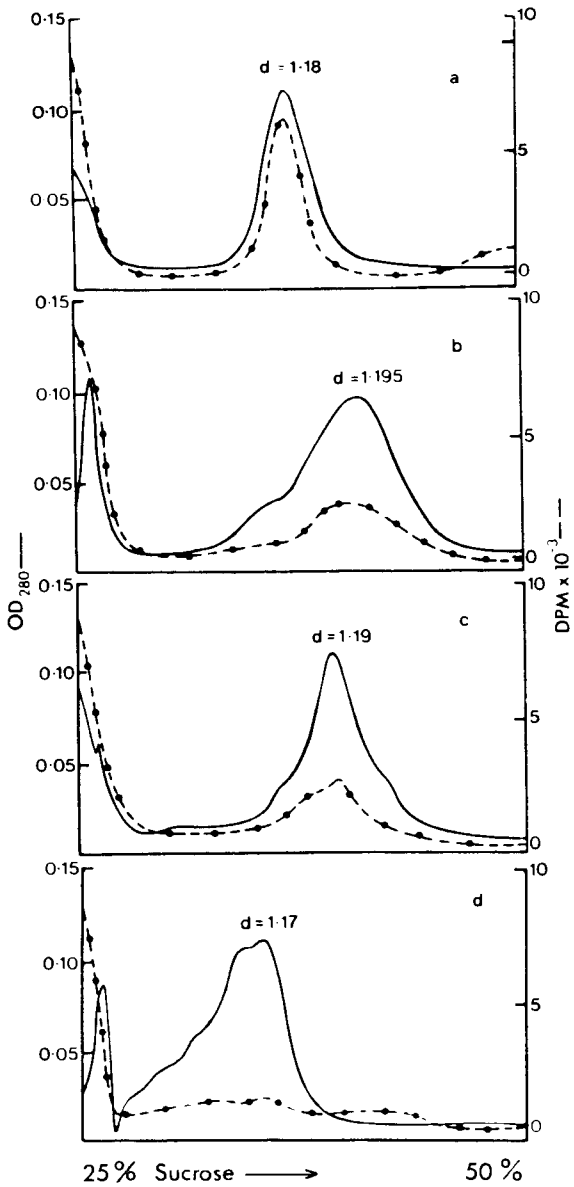

FIGURE 4.7 Sedimentation diagram of nuclear, microsomal and plasma membranes in a 25% to 50% sucrose gradient in TGA buffer after incubation with hormone receptor complex for 1 hour at +4°C. The amount of membrane in each incubation was identical with respect to protein. Centrifugation was for 3,5 hours at 170 000g.

a) Nuclear envelope

b) Rough endoplasmic reticulum

c) Smooth endoplasmic reticulum

d) Plasma membrane

The binding of the cytoplasmic TA-receptor by other membranes of the liver cell was considerably less than by the nuclear envelope. The plasma membrane exhibited less than 10% and the two endoplasmic membrane types less than 30% binding if compared to the nuclear envelope under identical conditions (Figure 4.7 and Table 4.3). The two endoplasmic membranes and the plasma membrane sedimented at sucrose densities different from that of the nuclear envelope. The nuclear envelope preparation on density gradient centrifugation appeared to be free from any significant contamination by the other membranes or radioactivity bound by the latter. Adenylate cyclase, a plasma membrane marker enzyme could not be detected in the envelope preparation, indicating negligible contamination by the plasma membrane (K. Grant, personal communication).

The nuclear envelope is thus enriched in acceptor sites for the TA-receptor complex compared to the other membranes of the liver cell. The presence of acceptors in the endoplasmic reticulum was not unexpected as this membrane is continuous with the outer nuclear membrane and the preparation may well contain a proportion of outer nuclear membrane. The differential binding of the TA-receptor to the variety of membrane types indicates that binding of the complex to the nuclear envelope is not simply a nonspecific TA-receptor/membrane association.

TABLE 4.3

BINDING OF CYTOPLASMIC (^{3}H)-TRIAMCINOLONE RECEPTOR COMPLEX TO NUCLEAR, ENDOPLASMIC AND PLASMA MEMBRANES

	dpm/100 µg PROTEIN
Nuclear envelope	6430
Rough endoplasmic reticulum	2140
Smooth endoplasmic reticulum	2064
Plasma membrane	542

Radioactivity bound by membranes was assayed after density centrifugation.

4.6 EFFECT OF LECTINS ON BINDING OF TA-RECEPTOR TO NUCLEAR ENVELOPE

As the bulk of nuclear envelope proteins are glycoproteins (3.2.3.3), the possibility that the TA-receptor complex binds the envelope via the carbohydrate moiety of an envelope glycoprotein was considered. If carbohydrate could be implicated in TA-receptor binding, the possibility of fractionation of envelope glycoproteins by lectin affinity chromatography could be considered. Envelope was therefore pre-incubated with 1 mg/ml Con A or LCH prior to incubation with TA-receptor complex. These lectins were chosen as the nuclear envelope is known to contain mannose and glucose, the respective ligands of the two lectins, as a large percentage of its carbohydrate content. After incubation with the lectin, the envelope was incubated with TA-receptor complex and fractionated on a 25 - 50% sucrose gradient. Results are shown in Figure 4.8.

Pre-incubation with either lectin did not diminish the binding of TA-receptor complex to the envelope. Glucose or mannose do thus not appear to be involved in the binding of the TA-receptor to the envelope. Envelope pre-incubated with Con A increased in density and banded further down the gradient at d = 1,19. This increased density was probably the result of a higher protein/lipid ratio in the envelope due to bound lectin. These results do not rule out carbohydrate as the binding determinant for the TA-receptor complex on the nuclear envelope as sugars other than glucose or mannose may be involved. In view of the above results, it is unlikely that glucose or mannose are directly implicated in the binding site.

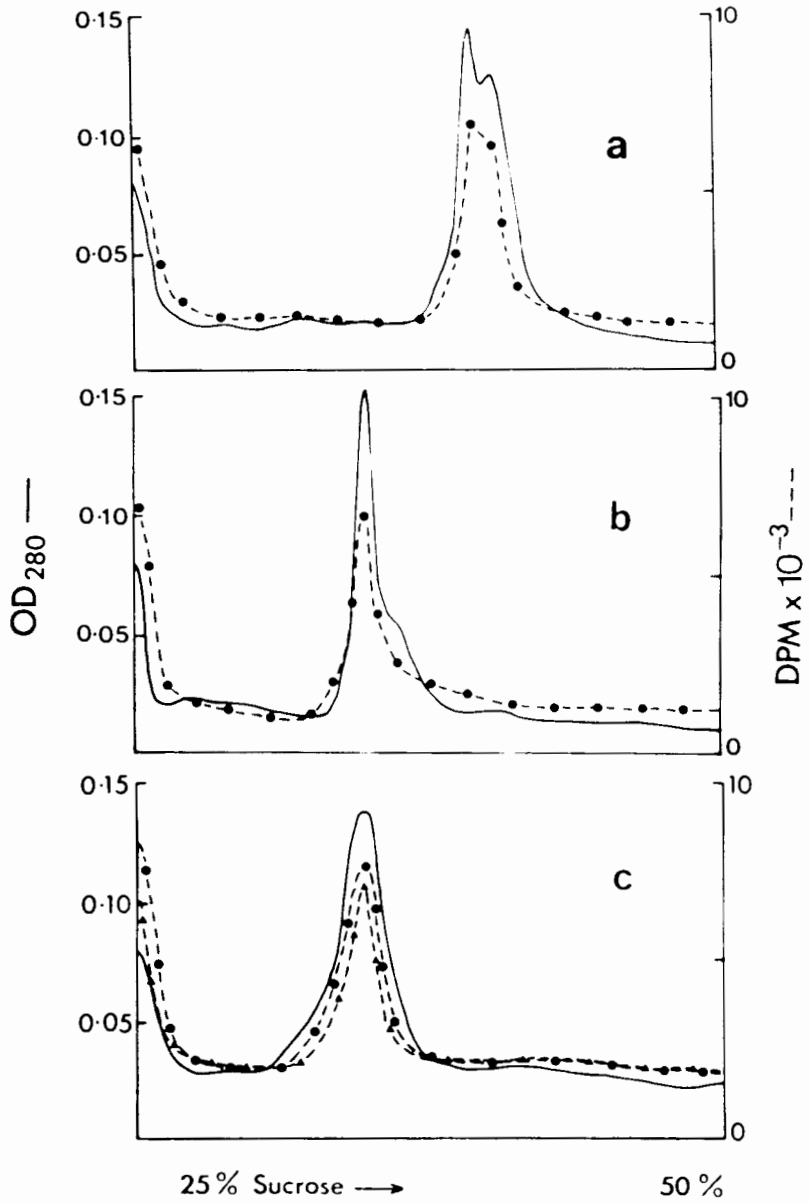

FIGURE 4.8 Sedimentation diagram of nuclear envelope incubated with TA-receptor complex.

(a) Nuclear envelope pre-incubated with Con A

(b) Nuclear envelope pre-incubated with Con A and 0,1 M α-D-methyl glucoside

(c) Nuclear envelope preincubated with LCH

●– –● in the absence of glucose

▲– –▲ in the presence of 0,1 M glucose

4.7 INTERACTION OF TA-RECEPTOR COMPLEX WITH THE NUCLEAR PROTEIN MATRIX

4.7.1 Introduction

A number of authors have reported the presence of proteinaceous steroid binding sites in the nuclear matrix (Barrack et al., 1977; Barrack and Coffey, 1980.) Binding sites for both estrogens and androgens have been found in the nuclear matrix (Barrack and Coffey, 1980). Whereas binding to the nuclear matrix does not necessarily preclude prior binding to the nuclear envelope, it does raise questions as to whether the observed binding of TA-receptor complex to the nuclear envelope is due to contamination of the envelope by elements of the nuclear matrix. Conversely, binding to the matrix might be due to the presence of nuclear envelope polypeptides in the matrix.

There are no reports in the literature about the extent of nuclear envelope contamination of the nuclear matrix other than that a residual nuclear envelope is located at the periphery of the matrix (Berezney and Coffey, 1977). Most authors assume that the bulk of membrane protein is removed by treatment with Triton X-100 and no attempts have been made to establish the proportion of matrix protein which derives from the envelope. The interrelationship between the nuclear envelope, the lamina and the nuclear matrix thus remains unclear. Berezney and Coffey (1980) have shown that a number of nuclear envelope enzymes persist in the matrix. Furthermore, the major matrix polypeptides, in the range of 60 000 - 70 000 daltons appear very similar to the major nuclear envelope polypeptides on SDS gel electrophoresis. The origin of matrix associated carbohydrate (5,5%) is as yet unestablished. Significantly, the carbohydrate content of the nuclear envelope is between 3 - 5% (Franke et al., 1976).

The protein and phospholipid content of the nuclear envelope as a percentage of total dry weight of components is remarkably similar to that of the endoplasmic reticulum (Franke et al., 1976). Significant contamination of nuclear envelopes with components of an intranuclear protein matrix would be expected to elevate the protein/lipid ratio when compared to a non-nuclear membrane such as the endoplasmic reticulum which is continuous with the outer nuclear membrane. This has not been observed.

Due to the fact that many of the functions previously associated with nuclear envelope are now being ascribed to the matrix (Berezney et al., 1980), it was decided not only to investigate whether the matrix bound TA-receptor complex, but also to evaluate the extent of contamination of the matrix by envelope polypeptides.

4.7.2 Isolation and Characterization of the Nuclear Protein Matrix

The isolation of the nuclear protein matrix from rat liver nuclei is described in detail in Methods (7.2.10) and outlined in Figure 4.9. The method of isolation used is identical to that employed by Berezney and Coffey (1974) and yielded a product with an overall composition similar to that obtained by them (Table 4.4). The matrix is an essentially proteinaceous structure in which nucleic acid or phospholipid are virtually absent. 5% of the matrix is carbohydrate which persists even after extensive dialysis to ensure removal of sucrose. Berezney and Coffey (1977) determined the carbohydrate content of the matrix, which they found to be 5,5%. They did not comment on the source or nature of the carbohydrate.

TABLE 4.4

COMPOSITION OF NUCLEAR PROTEIN MATRIX (PERCENT)

Protein	Phospholipid	Carbohydrate	DNA
$93,5 \pm 2,0$	$1,0 \pm 1,0$	$5,0 \pm 1,5$	$\leqslant 0,1$

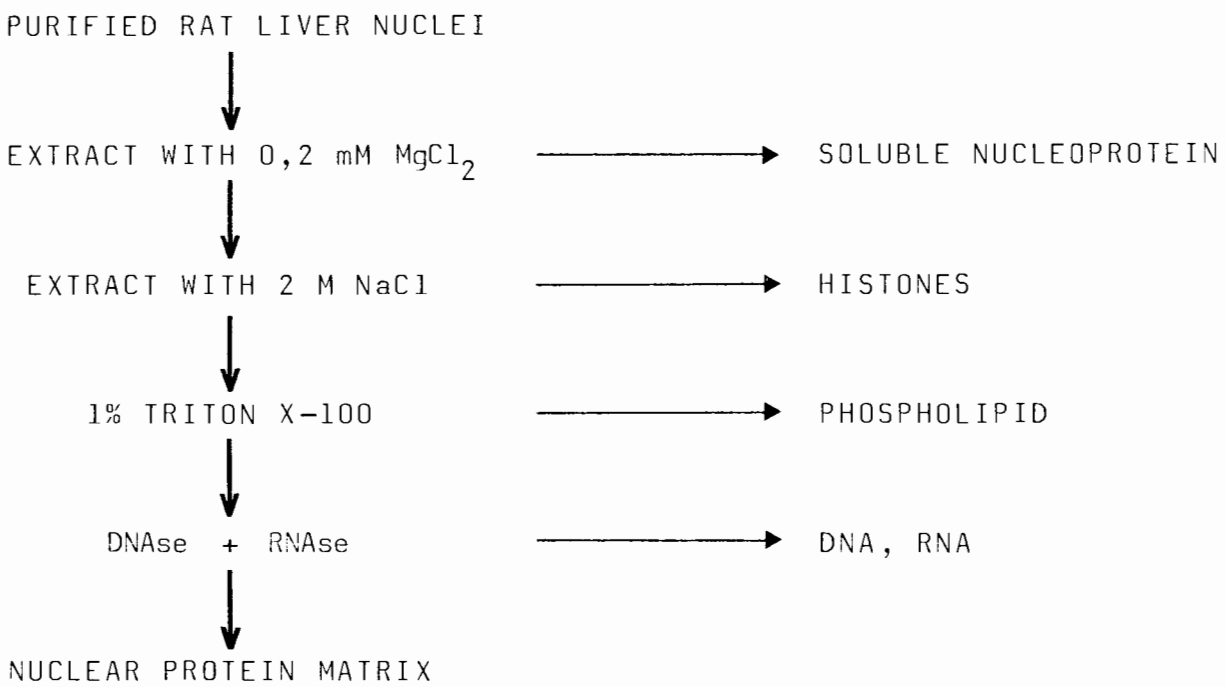

FIGURE 4.9 Isolation scheme for nuclear protein matrix

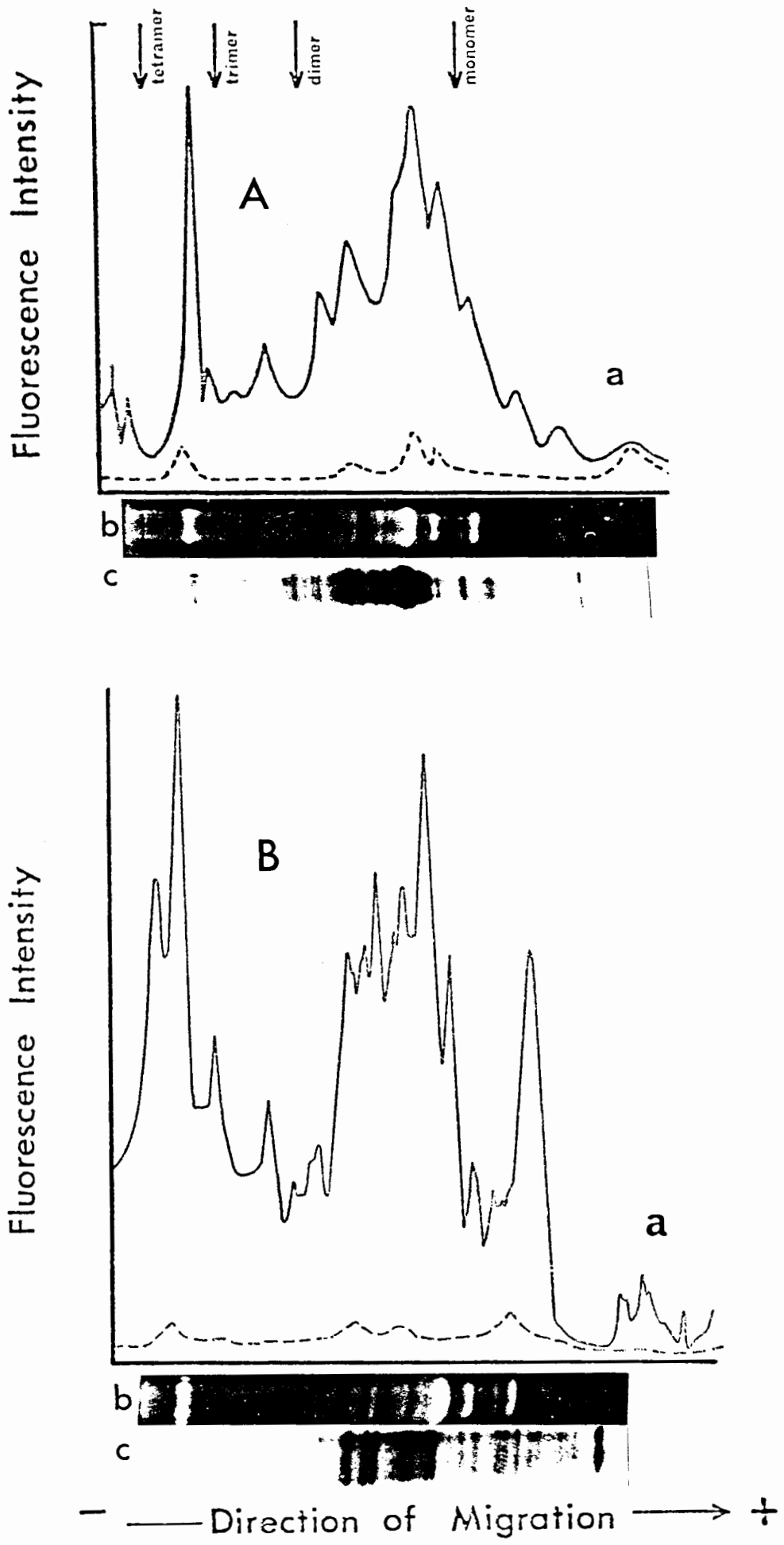

FIGURE 4.10 For legend, see following page.

FIGURE 4.10 SDS gel electrophoresis of nuclear envelope (A) and nuclear protein matrix (B). Gels were stained for protein with Coomassie Brilliant Blue and for glycoprotein with fluorescein labelled Con A.

(a) Scans of fluorescent gels
(———) binding in the absence of α-D-methyl mannoside
(-----) binding in the presence of α-D-methyl mannoside

(b) Gel stained with fluorescein Con A

(c) Gel stained with Coomassie Brilliant Blue

Arrows indicate molecular weight marker proteins:
Monomer = 53 000 daltons (BDH Product No. 44230)

TABLE 4.5

BINDING OF TA-RECEPTOR COMPLEX TO THE NUCLEAR PROTEIN MATRIX

		DPM BOUND
Nuclear protein matrix	100 µg	2 700
	200 µg	3 080
	300 µg	2 610
Blank		2 830

Each incubation contained 12 000 dpm. After incubation, samples were centrifuged and pellets washed twice in TGA buffer. Pellets were then solubilized in SDS and assayed for radioactivity. The blanks contained TA-receptor complex but no matrix.

4.7.3 SDS Gel Electrophoresis

The nuclear protein matrix was solubilized in SDS sample application buffer and subjected to SDS gel electrophoresis (7.3.1). Gels were stained for protein with Coomassie Brilliant Blue or glycoprotein with fluorescein Con A. Results are shown in Figure 4.10. A large number of bands bind Con A specifically, including a prominent high molecular weight band and a cluster of bands of lower molecular weight ($\pm$ 60 000 daltons) which are major components of the matrix (see Coomassie stained gel). The association of glycoproteins with the nuclear protein matrix has not previously been reported. The strong specific binding of Con A by these polypeptides is an indication of the probable presence of mannose or glucose. The localization of both these sugars in the nucleus has previously been shown to be exclusively in the nuclear envelope (Kawasaki and Yamashina, 1972).

Whereas the overall polypeptide distribution in the matrix and nuclear envelope shows differences on SDS gel electrophoresis, the glycoprotein distribution revealed by Con A binding is remarkably similar (Figure 4.10), although quantitative differences in bands are evident. The major polypeptides of the matrix all appear to be glycoproteins.

4.7.4 Incubation of Nuclear Protein Matrix with TA-Receptor Complex

Nuclear protein matrix was incubated with TA-receptor complex under the same conditions used for chromatin and nuclei (7.4.2). Increasing amounts of matrix were incubated with a constant amount of TA-receptor complex in 300 µl of TGA buffer for 1 hour at +4^{o}C. After the incubation the matrix was pelleted, washed in TGA buffer and assayed for radioactivity by extraction with 0,3M KCl. Nonspecific pelleting of radioactivity was carefully monitored. Results are presented in Table 4.7. No specific binding of TA-receptor was achieved, even in the presence of excess matrix. The nuclear acceptor site for TA-receptor complex is thus not retained in the matrix nor in residual envelope or nucleolar contaminants present in the matrix. The acceptor must therefore have been extracted by one of the steps employed to generate the matrix. Alternatively, one of the extraction steps may have altered the environment of the acceptor so as to render it inactive. This possibility is considered in Section 5.7.4.

4.8 ENVELOPE DERIVED PROTEINS IN THE NUCLEAR PROTEIN MATRIX

Isolation of nuclear envelopes using heparin produces only two fractions - an insoluble envelope and a soluble fraction containing DNA, protein and heparin (3.2.2). SDS gel electrophoresis of the protein component of the soluble fraction followed by staining with fluorescein Con A, fails to reveal the presence of any Con A binding polypeptides in the soluble fraction (Figure 3.12), confirming previous reports (Franke et al.,1976) on the membrane localization of nuclear glycoproteins. The presence of glycoproteins as major components of the nuclear protein matrix and the fact that their distribution on SDS gels is remarkably similar to the glycoprotein distribution of the nuclear envelope, indicates that either the envelope is contaminated with glycoproteins originating from the matrix or that envelope glycoproteins are major matrix components.

In order to investigate the extent of contamination of the matrix with envelope polypeptides a method was needed by which the selective labelling of the envelope in intact nuclei could be achieved. Richardson and Maddy (1980 a) have recently labelled intact nuclei with ^{125}I using solid phase lactoperoxidase. Over 90% of protein bound radioactivity was found associated with the nuclear envelope after fractionation of the nucleus. The suitability of this method was therefore investigated.

4.8.1 Iodination of Nuclei

Iodination of nuclei is described in detail in 7.3.9. Nuclei were prepared as previously described but special care was taken during isolation to avoid any damage to them. Nuclei pelleted through 2,3 M sucrose were gently resuspended in 0,25 M sucrose TKM with a glass rod. Large clumps of nuclei were allowed to settle under gravity and the suspension was filtered through 61µm mesh nylon gauze to remove smaller clumps. This yielded a final suspension free of aggregated nuclei. Solid phase lactoperoxidase was used as a generator of free radicles in order to ensure that only polypeptides on the outer surface of the nuclei became labelled. The Sepharose 4B beads to which the lactoperoxidase were coupled are far bigger than individual nuclei (Figure 4.11). After incubation the suspension was again checked for the presence of burst nuclei. If significant clumping was observed, the preparation was discarded.

After incubation, Sepharose 4B beads were removed by again passing the suspension through 61µm mesh nylon gauze. Nuclei were pelleted by centrifugation and washed three times in 0,25 M sucrose TKM or until the supernatant contained less than 2% of radioactivity found in the pellet. An average of 1% of input radioactivity was incorporated into protein. The specific activity of the labelled nuclei was on average 1×10^6 dpm/mg protein. SDS gel electrophoresis of whole labelled nuclei, followed by autoradioagraphy showed a number of labelled bands (Figure 4.12). The same labelling pattern was obtained from batch to batch of nuclei. Some labelling of histones occurs. This was expected due to the virtual impossibility of obtaining a preparation completely free of damaged or burst nuclei. Similar results were obtained by Richardson and Maddy (1980). However, extraction of histones from nuclei revealed that less than 5% of protein-bound radioactivity associated with this fraction, (see Table 4.6).

As a control, nuclei which had been purposefully burst by sonication were iodinated. SDS gel electrophoresis followed by autoradiography revealed extensive labelling of a large number of additional bands as well as a greater degree of labelling of histones (Figure 4.12). Histones extracted from these nuclei contained between 20% and 25% of bound radioactivity, (Table 4.6).

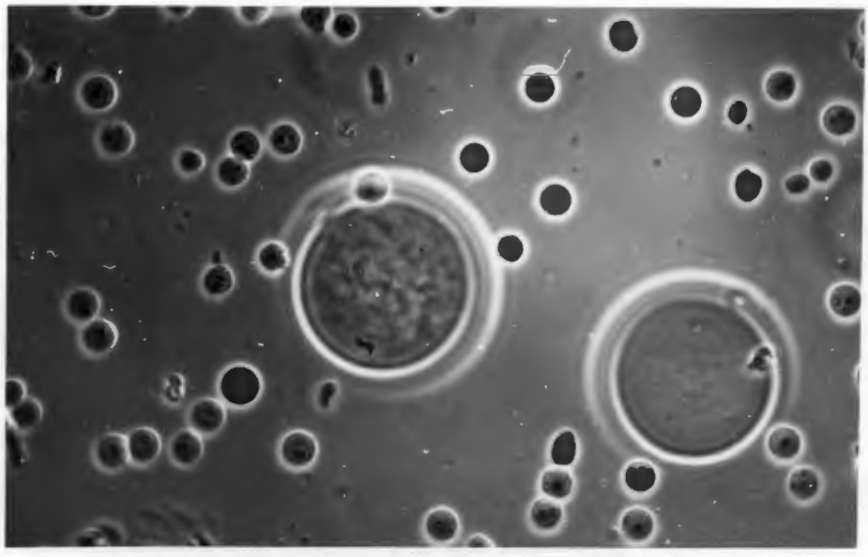

FIGURE 4.11 Purified rat liver nuclei and Peroxidase-Sepharose 4B beads.

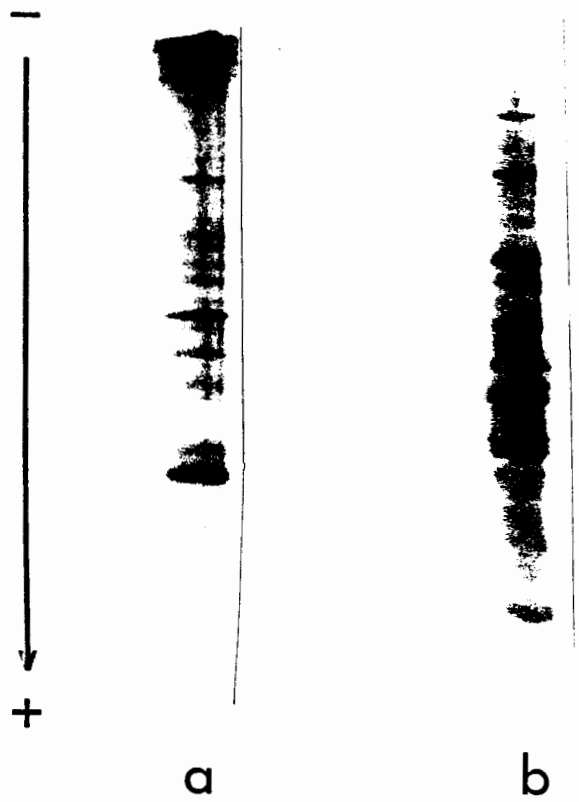

FIGURE 4.12 SDS gel electrophoresis followed by autoradioagraphy of
(a) ^{125}I labelled whole intact nuclei
(b) ^{125}I labelled sonicated nuclei

TABLE 4.6

DISTRIBUTION OF PROTEIN BOUND RADIOACTIVITY AFTER ISOLATION OF NUCLEAR ENVELOPE, NUCLEAR PROTEIN MATRIX OR HISTONES FROM ^{125}I LABELLED NUCLEI

	Input Radioactivity (dpm)	Bound Radioactivity (dpm)	Total Protein Bound Radioactivity (dpm)
Nuclear Envelope	1×10^8	9×10^5	$1{,}2 \times 10^6$
Nuclear Protein Matrix	1×10^8	5×10^5	$1{,}1 \times 10^6$
Total histone isolated from ^{125}I labelled nuclei	1×10^8	5×10^4	$1{,}0 \times 10^6$
Total histone isolated from ^{125}I labelled sonicated nuclei	1×10^8	$3{,}5 \times 10^5$	$1{,}6 \times 10^6$

Results represent an average of three determinations.

4.8.2 Fractionation of ^{125}I Labelled Nuclei

4.8.2.1 Isolation of Nuclear Envelopes

^{125}I labelled nuclei were added to unlabelled nuclei from four rats and nuclear envelopes were isolated as previously described (7.2.2). After pelleting envelopes by centrifugation, the solubilized heparin supernatant was dialysed to remove unbound iodine and the total bound counts in the supernatant and the nuclear envelope was determined (Table 4.6). Over 80% of bound radioactivity was found in the envelope fraction, indicating that mainly the outer surfaces of the nuclei were labelled during iodination. SDS gel electrophoresis of the envelopes followed by autoradiography revealed a labelling pattern essentially identical to that obtained for whole nuclei (Figure 4.13). Iodination of purified nuclear envelopes showed an increase in the number of labelled bands when compared to envelopes isolated from labelled nuclei (Figure 4.13). This was expected as in whole nuclei, membrane proteins exposed to the nucleoplasm would not be accessible to Sepharose beads and would therefore not become labelled.

4.8.2.2 Isolation of Nuclear Matrix

The nuclear matrix was isolated from iodinated nuclei together with unlabelled nuclei from four rats as carrier. Successive extractions were pooled, dialysed against water to remove unbound iodine and then counted for radioactivity. Acetylated dialysis tubing was used to ensure the inclusion of small proteins such as histones. Results are expressed in Table 4.6. A total of at least 50% of protein-bound radioactivity finally remained associated with the matrix. SDS gel electrophoresis of the matrix, followed by autoradiography, revealed a polypeptide labelling pattern similar to that obtained for whole nuclei and nuclear envelopes isolated from labelled nuclei (Figure 4.13).

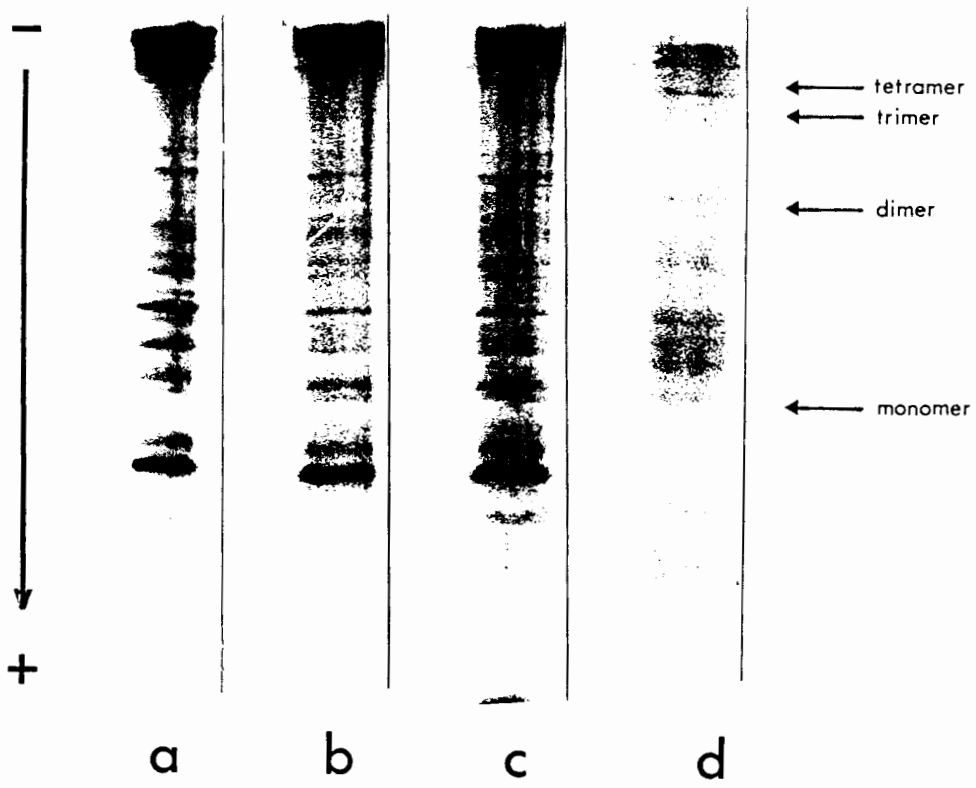

FIGURE 4.13

SDS gel electrophoresis, followed by autoradiography.

a) ^{125}I labelled whole nuclei

b) Nuclear envelopes isolated from ^{125}I labelled nuclei

c) Nuclear protein matrix isolated from ^{125}I labelled nuclei

d) ^{125}I labelled nuclear envelope

Arrows indicate molecular weight marker proteins:
monomer = 53 000 daltons (BDH Product No. 44230)

4.8.3 Discussion

The experiments reported here show that the nuclear envelope contains saturable high affinity acceptor sites for cytoplasmic TA-receptor complex which become labelled when intact nuclei interact "in vitro" with the activated TA-receptor complex. Furthermore these high affinity sites for the TA-receptor complex are also present in sucrose gradient purified chromatin as a contaminant of the latter with nuclear envelope fragments (Figure 4.5). and that in confirmation of the report by Jackson (1976) a large number of the polypeptide chains present in the chromatin non-histones result from this contamination of chromatin. The equilibrium constants in the order of 10^{-10} and 10^{-9} M for the interaction between the cytoplasmic steroid receptor and the envelope acceptor show that given the low intracellular hormone concentration, the nuclear envelope sites have those high affinity properties necessary for playing a biologically significant role in cytoplasmic-nuclear interaction. Calculated on the basis of 8.3 pg DNA per rat cell nucleus (Altman and Dittmer, 1964) the experiments reveal an average of 18 000 binding sites in the envelope per nucleus. The figure is of a similar order as those determined by Webster (Webster et al., 1976) who found 6 000 - 10 000 sites per nucleus for progesterone receptor in avian oviduct cells, Beato (Beato et al., 1974) who determined 15 000 sites per nucleus for dexamethasone in rat liver, and Bugany (Bugany and Beato, 1977) who found 5 000 - 10 000 sites per haploid genome in rat liver.

Of the other membranes tested for binding, the endoplasmic reticulum binds the TA-receptor with a specific activity one third of that found for the envelope. The plasma membrane binds with a specific activity one tenth of that found for the envelope. The binding sites observed in the endoplasmic reticulum could be due to the fact that this membrane system is continuous with the outer nuclear membrane. Nenci et al., (1980) have suggested that cytoplasmic steroid receptors may be loosely attached to the endoplasmic reticulum and released on interaction with the steroid or during a subsequent activation step.

Preincubation of envelopes with the lectins Con A and LCH did not prevent binding by the TA-receptor complex, indicating no direct role

for mannose or glucose in the acceptor site. The envelope is particularly enriched in protein-bound mannose which appears to be located only on the cisternal surfaces of the envelope (Virtanen and Wartiovaara 1976). Immunofluorescent and autoradiographic studies (Nenci et al., 1980; Sinha et al., 1973) located estradiol in the nuclear envelope but with insufficient resolution to determine preferential binding for inner or outer membrane.

The possibility that binding of the receptor complex to the nuclear envelope may have been caused by the 2% DNA in the membrane, can be discarded on two grounds. The binding characteristics of DNA as revealed by the binding of glucocorticoid receptor to unfractionated nuclei and chromatin are entirely different to those of the nuclear envelope (Figure 4.6a). Furthermore, the binding of steroid hormone receptor complexes by DNA is considerably diminished already at 0,1 M salt (Rousseau et al., 1975; Milgrom et al., 1976), the concentration of sodium chloride in the binding test (see Methods 7.4.2). That binding in these experiments does not decrease at 0,15 M NaCl (Figure 4.3) but is only abolished at higher salt concentration indicates that the nuclear envelope represents a nuclear subfraction enriched in specific, high affinity acceptor sites other than DNA for cytoplasmic steroid hormone receptor complex.

The ability of the nuclear envelope to bind cytoplasmic TA-receptor complex may explain why the nuclear matrix has been reported to retain labelled steroid hormone after injection into rats (Barrack et al., 1977). The nuclear matrix consists of a large number not only of intranuclear chromosomal and nucleosomal proteins but also to a considerable extent of residual nuclear envelope proteins insoluble under the conditions of matrix isolation (Berezney and Coffey, 1976). Similarly, the binding of cytoplasmic steroid hormone receptor by non-histone (O'Malley et al., 1977) may be possibly partly due to the high degree of contamination of chromatin with nuclear envelope proteins found in these experiments and also reported earlier (Jackson, 1976). The results presented here raise the question as to whether the matrix can be regarded as a nuclear structure distinct from the nuclear envelope. Furthermore they suggest that envelope derived proteins and glycoproteins may comprise the bulk of total matrix protein.

Radioiodination of whole nuclei using solid phase lactoperoxidase resulted in the association of over 80% of bound radioactivity with the nuclear envelope. Similar results were obtained by Richardson and Maddy (1980). The bulk of this radioactivity was associated with the protein, rather than lipid fraction of the envelope. As the envelope comprises less than 10% of total nuclear protein, the iodination must have occurred predominantly on the outer surface of the nuclei. The association of iodinated proteins with the nuclear matrix isolated from iodinated nuclei must be mainly due to components derived from the nuclear envelope. The fact that such a large proportion of total protein-bound radioactivity associates with the matrix is a strong indication that at least 50% if not more of nuclear envelope proteins, co-isolate with the matrix, despite extraction with 2 M NaCl and Triton X-100. The similarity in glycoprotein composition of matrix and envelope is striking, particularly in the fact that by criteria of SDS gel electrophoresis, almost every Con A binding envelope glycoprotein is also present in the matrix.

The binding of lectins by the matrix has been noted by Sevaljevic et al., (1981) and the presence of carbohydrate confirmed by Berezney and Coffey (1977) who found that carbohydrate comprised about 5% of the matrix by weight. This is significant in view of the fact that the bulk of nuclear carbohydrate can be accounted for by the nuclear envelope. 100% of mannose and 60% of glucosamine were found in the membrane which contained 4% carbohydrate by weight, a figure very similar to that obtained for the matrix. The results presented here confirm the presence of protein linked carbohydrate in both envelope and matrix in similar proportions. The recovery of nuclear protein in the envelope and matrix is likewise very similar, varying between 10 and 20% depending on the method of isolation used (Berezney and Coffey, (1977); Kaufmann et al.,(1981); Kashnig and Kasper (1969); Kay et al.,(1972). Kaufmann et al.,(1981) found that the protein recovery in nuclear ghosts prepared from rat liver did not differ substantially from nuclear matrices prepared from the same nuclei. They found further that the intranuclear matrix network observed by electron microscopy could be obtained by standard procedures from freshly isolated rat liver nuclei only in the presence of a disulfide promoting agent such as sodium tetrathionate. Nuclei which had stood for 12 - 24 hours at $+4^{o}C$ prior to matrix isolation also showed the intranuclear matrix network. However storage of these nuclei in the presence of 10 mM iodoacetamide, an agent which minimizes the oxidation of protein sulfhydryl

groups, prior to matrix isolation, yielded only nuclear ghosts devoid of an intranuclear matrix network. Lam and Kasper (1979b) have recently described disulfide-crosslinked oligomers of the major rat liver nuclear envelope proteins. Shelton et al.,(1982) have observed large polymers of disulfide linked envelope proteins. Cross-linking can be blocked by treating nuclei with N - ethylmaleimide (Shelton and Cochran, 1978).

In a recent paper by Galcheva-Gargova et al.,(1982), the authors suggest that the integrity and stability of the matrix is acquired in the course of isolation and is linked to chromatin condensation. After nuclei are swollen in the presence of EDTA and then digested with DNase, structures devoid of internal network are obtained. If nuclei are swollen, exposed to Mg^{++} and then digested with DNase, an internal network is obtained. The presence of Mg^{++} maintains the chromatin in a condensed state. The authors suggest that the matrix is produced by irreversible aggregation of nonhistone chromatin proteins on digestion of the DNA.

These results, together with those presented here, indicate that far from being a minor contaminant of the matrix, envelope derived proteins do contribute a large proportion of matrix protein. It is therefore clearly too early to suggest that functions attributed to the matrix have an intranuclear location. Given the preponderance of envelope proteins in the matrix, speculation as to the function of the matrix as an entity distinct from the nuclear envelope is clearly premature.

PART 5

FRACTIONATION AND RECONSTITUTION OF NUCLEAR ENVELOPE PROTEINS

INTRODUCTION

Having established the presence of acceptor sites for TA-receptor complex in the nuclear envelope, the question of the location of these sites in the envelope was addressed. If the envelope is merely involved in the transport of the steroid hormone receptor complex into the nucleus, then the pore complex would be the obvious candidate as the acceptor. As the binding experiments conducted do not distinguish binding to cytoplasmic or nucleoplasmic faces of the membrane, the possibility must be considered that the steroid hormone receptor enters the nucleus passively through a pore and binds to nucleoplasmic surface of the inner nuclear membrane, i.e. the lamina.

Clearly a technique in which the steroid hormone receptor could be covalently linked to its envelope acceptor site would yield maximum information. However, this technique requires a homogenous preparation of TA-receptor complex rather than a partially pure preparation. Furthermore, the steroid itself is noncovalently bound to its receptor and the complex is highly labile. Affinity labelling of a number of steroids to their receptors has been performed but the biological activity of the products in most cases has not been established.

In order to establish the location of the acceptor in the envelope, it was decided to attempt fractionation of the envelope and to check the various fractions for acceptor activity. As the natural environment of envelope proteins is a lipid bilayer it was clear that if fractionation was to involve extraction of proteins from the bilayer, reconstitution with lipid would be necessary prior to testing for acceptor activity.

As a first attempt to identify the nuclear envelope acceptor for the TA-receptor complex, it was necessary to establish whether it was possible to solubilize, fractionate and reconstitute the envelope components and then test the reconstituted membrane for acceptor activity. The choice of a suitable solubilizing agent for the membrane was important. Nonionic detergents often provide selective solubilization of membrane components but are difficult to remove. Harsher detergents such as SDS solubilize membranes adequately, but denature the proteins. Such detergents are also difficult to remove. A variety of organic solvents such as butanol, methanol and chloroform will solubilize the lipid components of membranes, but may denature the proteins.

2-chloroethanol is a unique solvent which solubilizes membrane proteins, lipids and most small contaminants. Both proteins and lipids are maintained in a monomeric state and can be effectively separated by gel permeation chromatography on Sephadex LH 20 as first shown by Zahler and Wallach (1967). More than most other organic solvents, 2 - chloroethanol promotes the formation of the right-handed α - helix. This is true also for membrane proteins (Lenard and Singer, 1966). Promotion of α-helix formation implies formation of more or maximal intra-chain hydrogen bonds i.e. minimal scrambling. Most agents used in membrane studies, such as SDS, urea or guanidine HCl induce unwinding of the peptide chain. Many enzymes can be put through a chloroethanol cycle without loss of activity (Wallach and Winzler, 1974). Agents such as SDS tend to promote structures (especially in basic polypeptides) which can generate cross linking between peptide chains and hinder the fractionation of monomers.

2-chloroethanol appears to be somewhat unstable, tending to release HCl. The freshly distilled solvent is transparent to 230 nm and has an apparent pH of 1,7. It was originally thought that an acid pH was necessary for the solvent action of 2-chloroethanol, but 80% 2-chloroethanol, 20% 50 mM phosphate pH 7,4 still solubilizes erythrocyte ghosts effectively (Wallach and Winzler, 1974). The suitability of 2-chloroethanol as a solvent for the nuclear envelope was therefore investigated.

Methodology for the reconstitution from 2-chloroethanol of membrane protein and lipid has been developed by Zahler and Weibel (1970). They solubilized human erythrocyte membranes in chloroethanol:water (9:1) pH 2,0 and separated lipid and protein components of the membrane by chromatography on Sephadex LH 20 at $0^{o}C$. Lipid and protein fractions were recombined and dialysed against 10 mM Tris (pH 7,6), 10 mM $CaCl_2$, resulting in the formation of a white precipitate of recombined material. Electron microscopy of original membranes and recombined material revealed identical structures. Both were trilaminar with the same overall thickness of 7 - 8 nm and had an identical granular substructure of dense layers. The protein components of both recombined and native membranes were indistinguishable by SDS gel electrophoresis. By a number of criteria the products of reconstitution of erythrocyte membrane components from chloroethanol thus appeared identical to native erythrocyte membrane.

On the basis of these results, it was therefore decided to follow the reconstitution protocol of Zahler and Weibel (1970). Unfortunately, during the course of this study a facility for electron microscopic examination of reconstituted material has not been available, and reconstituted fractions have been analysed by sucrose gradient centrifugation and SDS gel electrophoresis. In the absence of positive identification of a reformed bilayer, the reconstituted fractions are referred to here as "reconstituted material"or "reconstituted complex" rather than "reconstituted membrane".

5.1 SOLUBILIZATION OF THE NUCLEAR ENVELOPE IN 2-CHLOROETHANOL

All work was performed at +4°C to minimize enzyme activity. The envelope was dissolved in 2-chloroethanol:0,05 M phosphate, pH 7,0 in the concentration range 1 - 5 mg/ml protein. At these concentrations centrifugation at 50 000 g for 1 hour did not produce any precipitate and the membrane was presumed solubilized in toto.

Figure 5.1 shows a scan of total nuclear envelope solubilized in 2-chloroethanol. The spectrum is a typical protein spectrum, virtually identical to that obtained when the envelope is solubilized in 1% SDS.

5.2 RECONSTITUTION OF TOTAL NUCLEAR ENVELOPE FROM 2-CHLOROETHANOL

In order to establish whether it was possible to reconstitute solubilized nuclear envelope from 2-chloroethanol, 0,5 mg of nuclear envelope was solubilized in 1 ml 2-chloroethanol:0,05 M phosphate, pH 7,0 (9:1) at +4°C and dialysed against 100 ml of 0,1 M NaCl, 0,01 M Na phosphate, pH 7,5 overnight. A white suspension formed in the dialysis bag and this was pelleted by centrifugation at 50 000 g for 30 minutes. The resulting supernatant was essentially protein free. It contained only 5% of the original amount of protein as estimated by absorption at 280 nm.

The pellet was resuspended in 0,05 M phosphate pH 7,0, applied to a 25 - 50% sucrose gradient, centrifuged for 3 hours at 170 000 g and analysed as previously described (see 7.2.2). Results are expressed in Figure 5.2.

The reconstituted envelope had a slightly higher density than native nuclear envelope. It sedimented to a sucrose density of 1,195 on the gradient. The increased density is probably due to the protein:lipid ratio of the reconstituted envelope being slightly greater than that of the isolated nuclear envelope. The presence of a small amount of lipid at the top of the gradient supports this conclusion. Reconstitution was not selective for any particular envelope protein. The polypeptide pattern obtained on SDS gel electrophoresis of the reconstituted envelope was virtually identical to that obtained for native envelope, apart from quantitative differences in bands (Figure 5.3) indicating successful

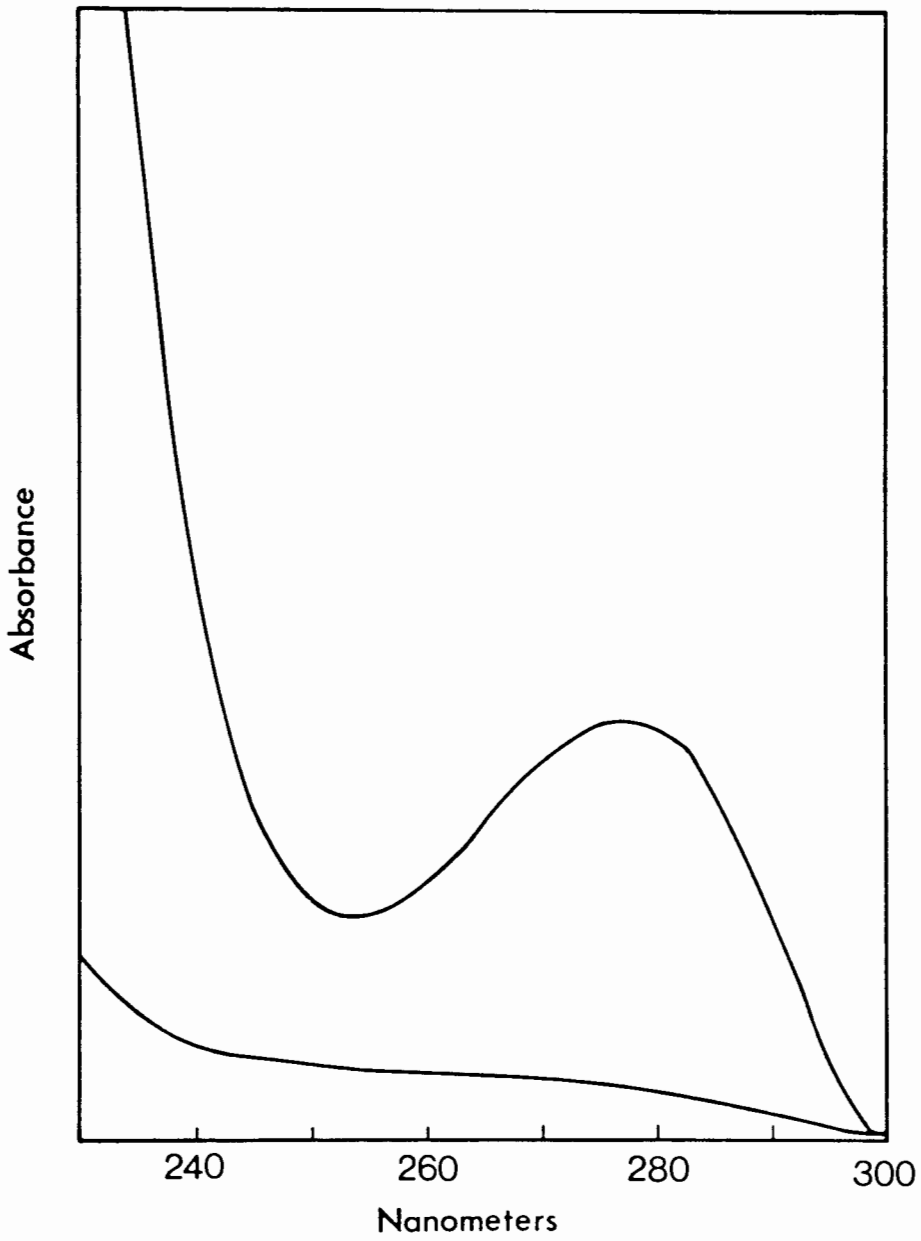

FIGURE 5.1 U.V. Spectrum of total nuclear envelope solubilized in 2-chloroethanol:0,05 M Na phosphate pH 7,5 (9:1).

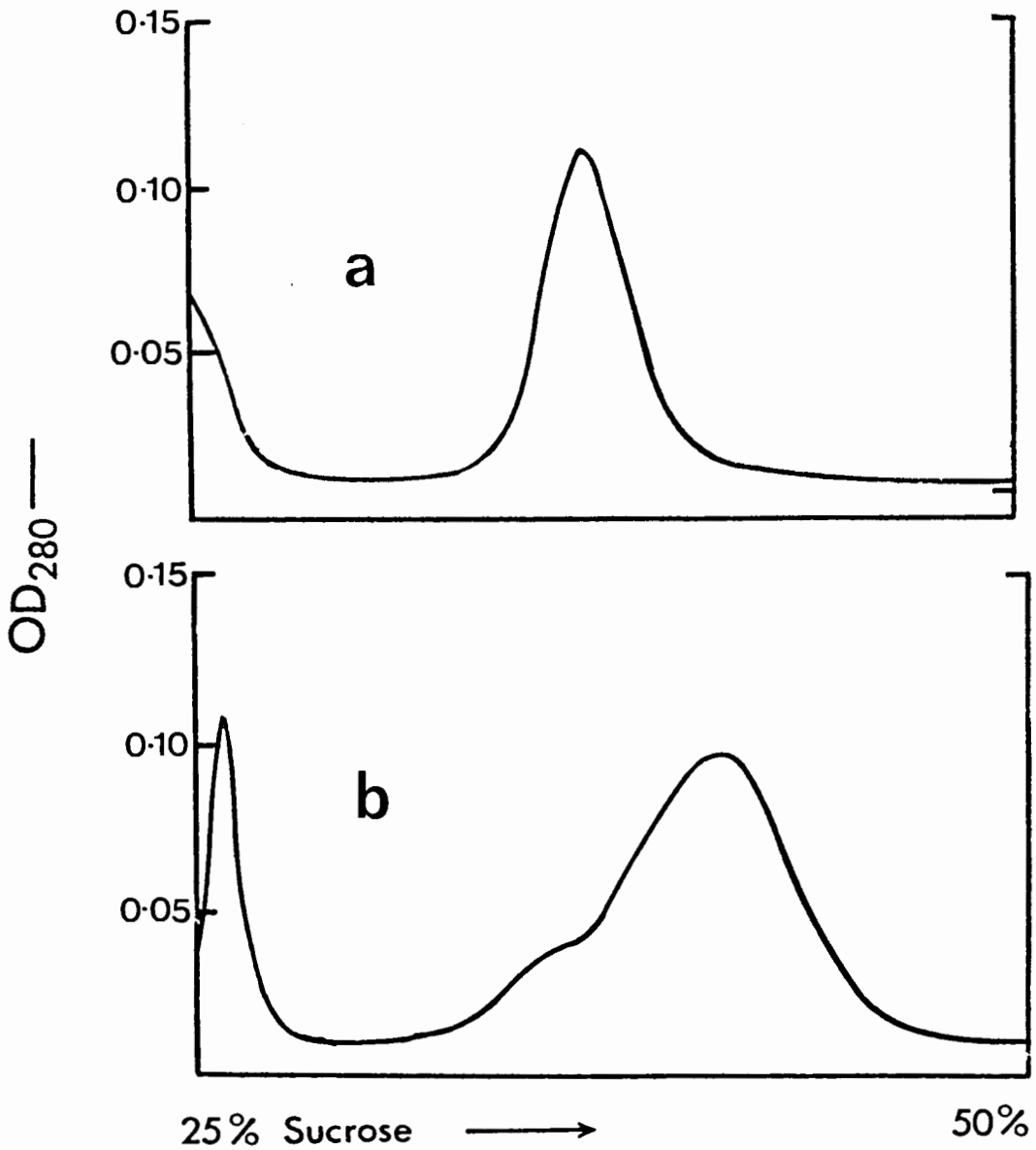

FIGURE 5.2 Sedimentation diagrams of a total nuclear envelope.

(a) Total nuclear envelope

(b) Total nuclear envelope reconstituted from chloroethanol:0,05 M Na phosphate, pH 7,5 (9:1) as described in 5.2.

Centrifugation was for 3 hours at 170 000 g.

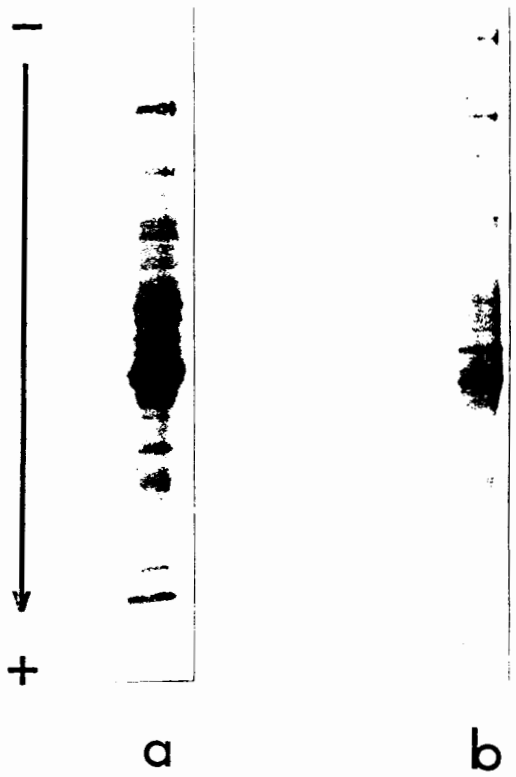

FIGURE 5.3 SDS gel electrophoresis of

(a) Total nuclear envelope

(b) Nuclear envelope reconstituted as described in 5.2.

Electrophoresis was for 3 hours on a 10% polyacrylamide gel.

reconstitution of all envelope polypeptides. No protein was found at the top of the gradient, but a small pellet representing about 10% of the total protein was obtained.

These results are in accordance with the findings of Zahler and Weibel (1970) who reconstituted chloroethanol solubilized erythrocyte membranes by dialysis against 10 mM Tris pH 7,6. They obtained a reconstituted membrane which had a slightly higher density than native membrane but contained essentially all the proteins and lipids apart from the glycolipids. Furthermore, electron microscopy of their reconstituted material revealed reformed membranes of more of less the same structure as native membranes.

To establish whether the reconstituted envelope retained the ability to bind TA-receptor complex, an aliquot (150 µg protein) was incubated with TA-receptor and the binding analysed on a 25 - 50% sucrose gradient as previously described (See 4.2). The results are expressed in Figure 5.4. The reconstituted envelope retained its ability to bind TA-receptor complex. The envelope acceptor is thus able to withstand exposure to 2-chloroethanol. Inclusion of 0,3 M KCl in the gradient released all envelope-bound radioactivity (Figure 5.4). Quantitation of binding revealed that the reconstituted envelope had a lower specific activity when compared to native envelope (Table 5.1). The reconstituted envelope bound 50% less TA-receptor complex when compared to native envelope. This partial loss of binding activity by the reconstituted envelope is probably at least partially due to the formation of closed vesicles during reconstitution which have a proportion of acceptor sites located on the inside of the membrane and hence not available for binding TA-receptor complex. Alternatively, extraction and reconstitution procedures may themselves cause loss of acceptor activity through inactivation or denaturation of acceptor protein. However, these results show that in principle reassembly of solubilized nuclear envelope from 2-chloroethanol is possible and that in reassembled envelope, acceptors for the TA-receptor complex are present in the correct orientation and are still active in binding the complex.

In order to establish whether native envelope lipid was essential for successful reconstitution and whether TA-receptor binding was dependent on the presence of native lipid it was decided to pursue fractionation of the lipid and protein components of the membrane and attempt reconstitution of lipid free protein with both total envelope lipid and a single lipid species such as lecithin.

TABLE 5.1

BINDING OF TA-RECEPTOR COMPLEX TO RECONSTITUTED TOTAL NUCLEAR ENVELOPE

FRACTION	DPM BOUND PER 100 µg PROTEIN
Nuclear envelope	2580
Reconstituted total nuclear envelope	1520

Results represent an average of three determinations.

Input radioactivity was 20 000 dpm. 100 µg membrane protein was used per incubation. Incubation conditions are described in 7.4.1.

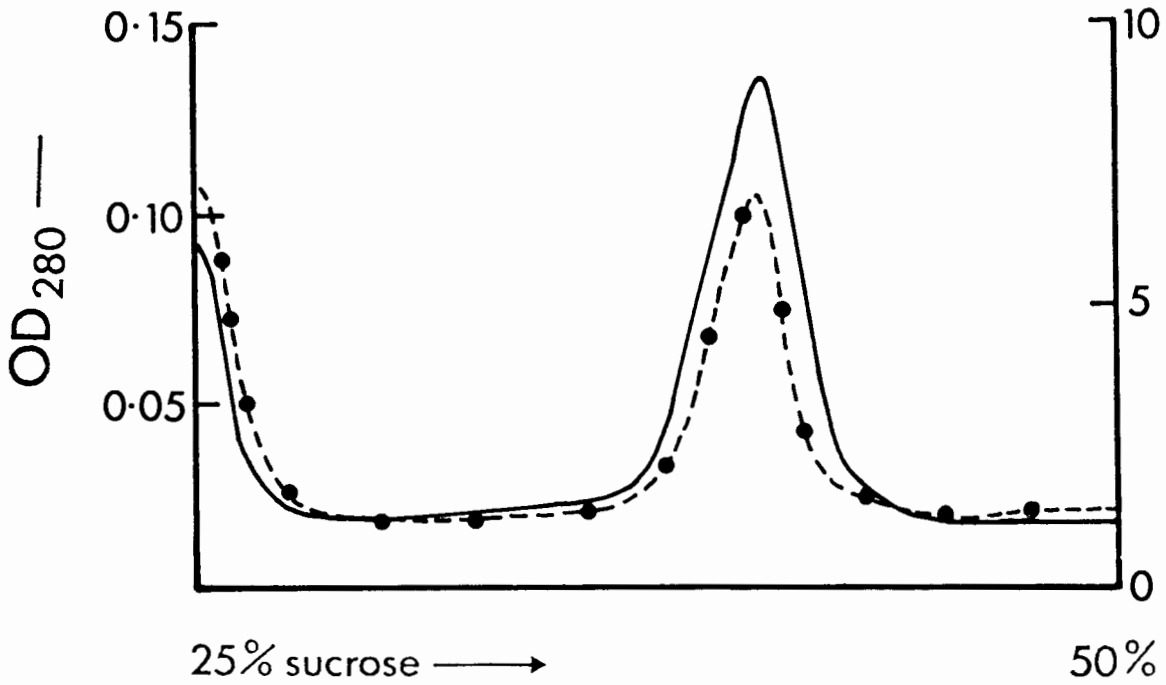

FIGURE 5.4 Sedimentation diagram of reconstituted total nuclear envelope after incubation with TA-receptor complex. (See 5.2)

Centrifugation was for 3 hours at 170 000g.

5.3 FRACTIONATION OF THE NUCLEAR ENVELOPE ON SEPHADEX LH 20

Sephadex LH 20 was chosen as a good matrix for the separation of nuclear envelope phospholipid from total envelope protein. This matrix has been previously successfully used with chloroethanol to separate protein and phospholipid from solubilized plasma membrane (Zahler and Wallach, 1967). Nuclear envelope (5 mg protein) was solubilized in 1 ml of chloroethanol: 0,05 M phosphate pH 7,5 (9:1) and applied to a column of Sephadex LH 20 equilibrated in the same solvent. The column dimensions were 1 m x 1,0 cm. A typical elution profile is shown in Figure 5.5. Protein was monitored by absorption at 280 nm and phospholipid detected as phosphorus (see 7.3.5). Complete separation of protein and phospholipid was achieved on this column. Phospholipids chromatographed as monomers in the inner volume while protein eluted in the outer volume. The column was run at $+4^{o}C$ to minimize proteolysis.

During pilot reconstitution experiments it soon became evident that to routinely monitor the extent of incorporation of fractionated nuclear envelope protein into reconstituted membranes, a relatively large amount of protein would have to be used to remain within the limits of detection by the Folin-Lowry procedure. Therefore it was decided to radioiodinate the envelope prior to fractionation so that reconstitution could be routinely monitored by extent of incorporation of radioactivity into the reconstituted membrane. The membrane was iodinated using solid phase lactoperoxidase as described in 7.3.9. All the major envelope polypeptides are labelled by this procedure (Figure 4.13). Iodinated envelopes were solubilized in 2-chloroethanol and fractionated on Sephadex LH 20. Fractions were monitored for radioactivity on a Packard Autogamma counter. Results are expressed in Figure 5.6. The fractionation obtained was similar to that obtained with unlabelled envelope (Figure 5.5). Non protein bound iodine eluted with the phospholipid in the inner column and was well separated from the protein fraction. The reason for the presence of two peaks in the outer volume was not investigated.

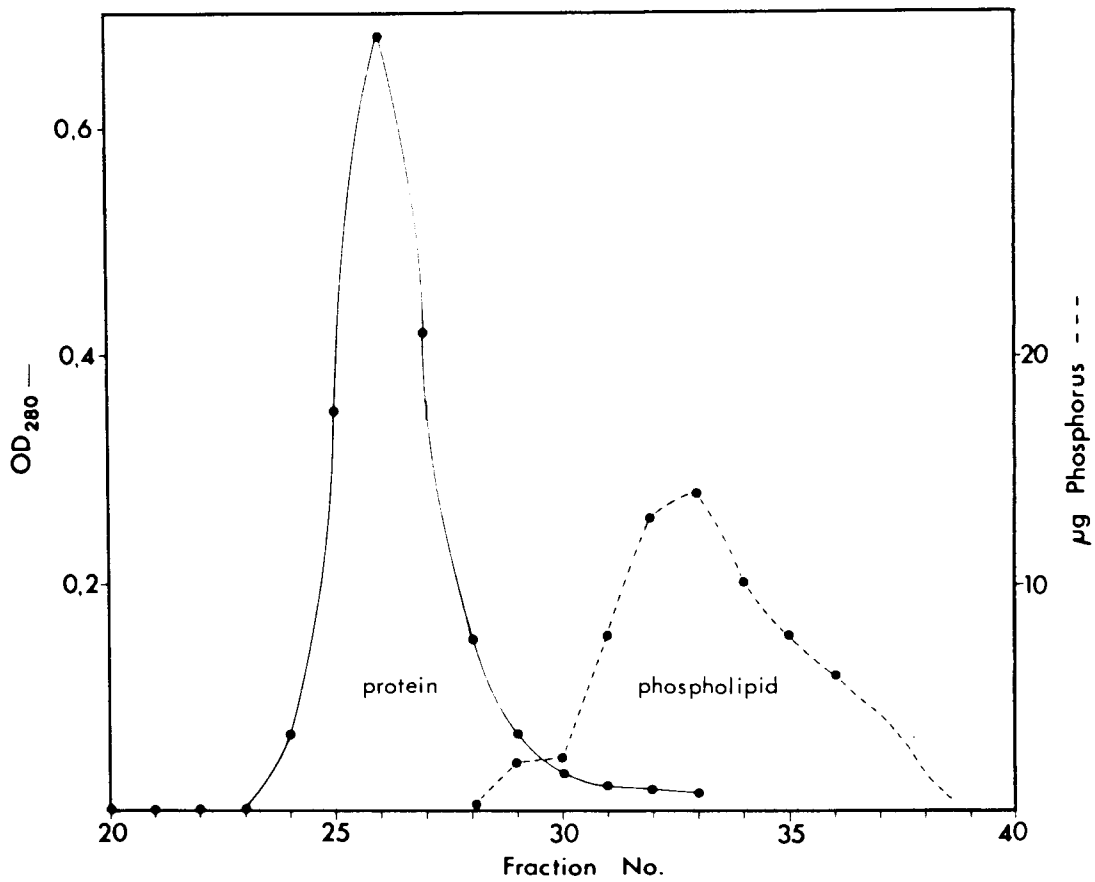

FIGURE 5.5 Fractionation of nuclear envelope on Sephadex LH 20 in 2-chloroethanol:0,05 M phosphate, pH 7,5 (9:1). Fraction volume = 1 ml. Phospholipid was assayed as phosphorus (7.3.5) and protein by absorbance at 280 nm.

----- µg phosphorus

——— OD_{280}

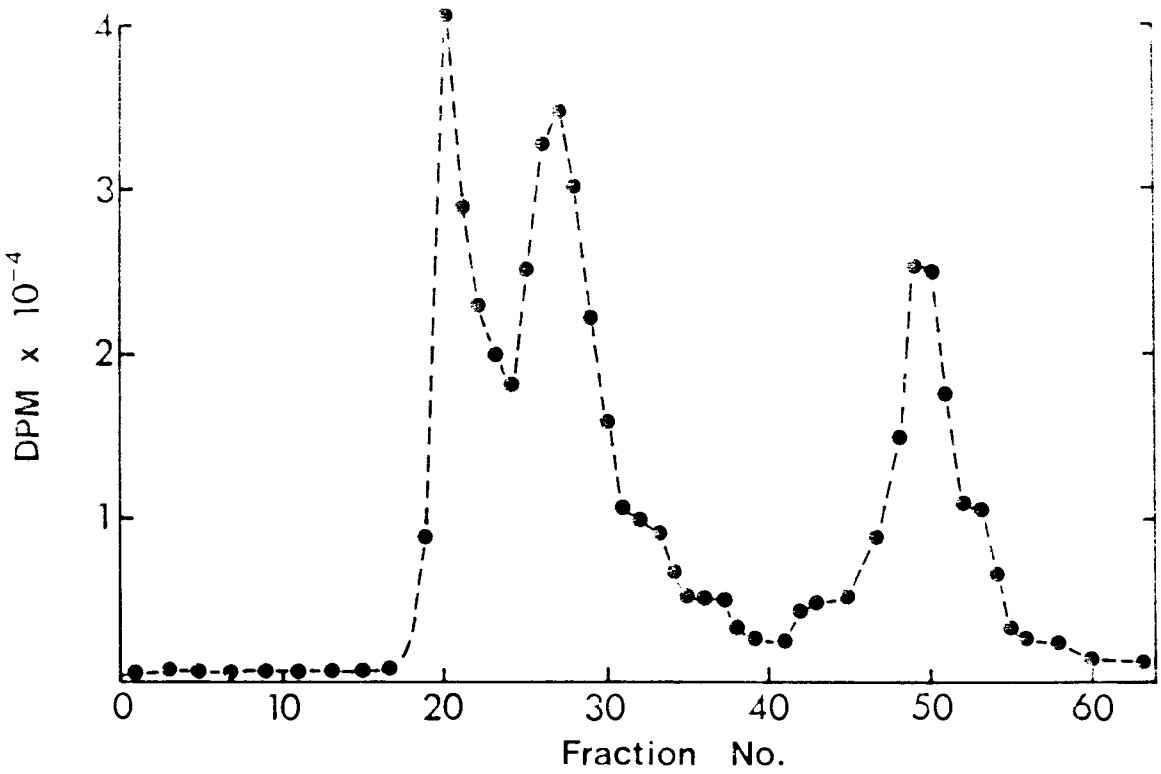

FIGURE 5.6 Fractionation of ^{125}I labelled nuclear envelope on Sephadex LH 20 in 2-chloroethanol:0,05 M phosphate, pH 7,5 (9:1) as described in 5.3.

5.4 RECONSTITUTION OF LIPID FREE NUCLEAR ENVELOPE POLYPEPTIDES

5.4.1 Reconstitution with Total Nuclear Envelope Lipid

Total nuclear envelope lipid was extracted from purified envelopes using chloroform:methanol (2:1) (See 7.2.11). 1 mg of lipid was dried under nitrogen. To this was added 300 µl of a 1 mg/ml solution of lipid free total nuclear envelope protein in 2-chloroethanol. The solution was immediately dialysed against 500 mls of 0,1 M NaCl, 0,01 M phosphate, pH 7,5 at +4°C overnight.

After dialysis the contents of the dialysis bag were layered over a 25 - 50% gradient and centrifuged for 2,5 hours at 38 K rpm in a SW40 Ti Rotor. Analysis of the gradient revealed a single band at a position corresponding to a density of 1,18 (Figure 5.7), close to that of native nuclear envelope (d = 1,17). A small pellet was observed as well as an amount of material at the top of the gradient. The three fractions were collected and analysed by SDS gel electrophoresis. Results in Figure 5.8 show that the reconstituted material has a similar polypeptide composition to isolated nuclear envelopes. Although some protein pelleted, none was observed at the top of the gradient. When the reconstitution was performed with iodinated envelope proteins, over 70% of the radioactivity was found associated with the reconstituted material (Figure 5.7) indicating that under these conditions over 70% of lipid free envelope protein reassociates with envelope lipid after reconstitution. Similar results were obtained by Kramer et al.(1972).

5.4.2 Reconstitution with Lecithin

The procedure adopted for the reconstitution of chloroethanol solubilized nuclear envelope proteins with lecithin is described in Methods (7.4.5). Aliquots of lipid free nuclear envelope protein used in reconstitution experiments were obtained after fractionation of the envelope on Sephadex LH20. Initially, increasing amounts of total nuclear envelope protein were reconstituted with 3 mg of lecithin from 300 µl of chloroethanol by dialysis against 0.1 M NaCl, 0,01M phosphate pH 7,5. The reconstituted material was analysed by sucrose gradient centrifugation. Reconstitution

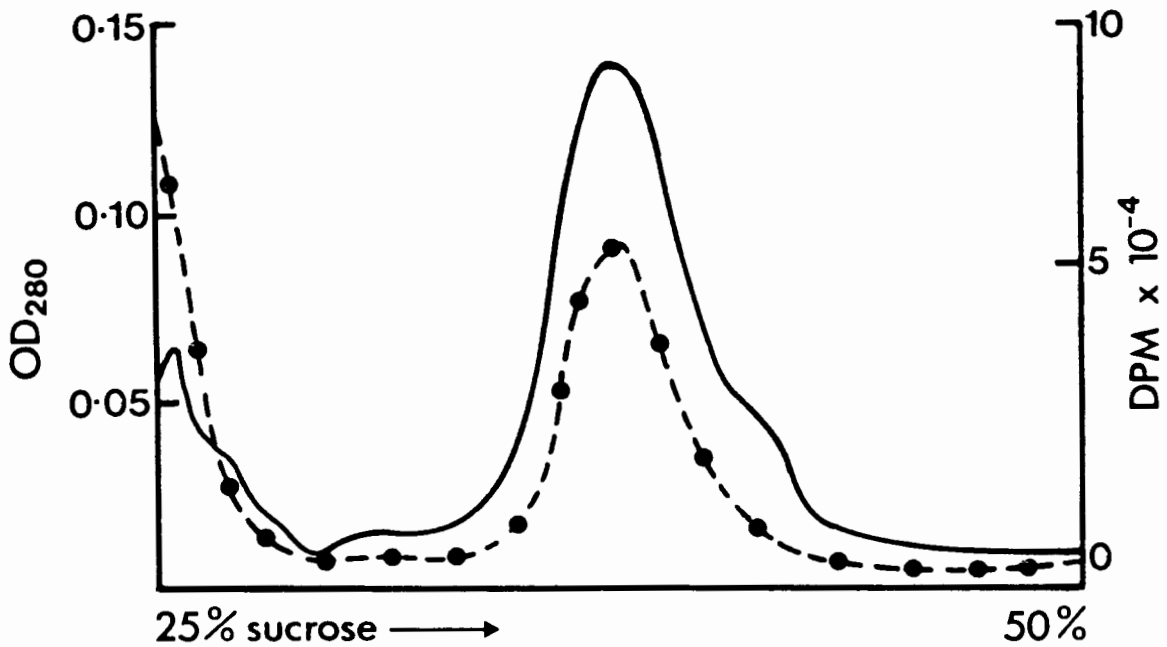

FIGURE 5.7 Reconstitution of lipid free total nuclear envelope protein with envelope lipid under conditions described in 5.4.1. Reconstituted material was centrifuged on a 25 - 50% sucrose gradient for 3 hours at 170 000 g.

(a) Unlabelled envelope protein.
(b) ^{125}I labelled envelope protein.

with lecithin proved less straightforward than reconstitution with total envelope lipid.

The final products obtained after reconstitution depended chiefly on two parameters: the protein/lipid ratio and the concentration of the components used in the reconstitution. A lipid/protein ratio of less than 10:1 produced on gradient centrifugation, an essentially protein free fraction of lipid at the top of the gradient (Figure 5.9A) and an aggregated protein component which pelleted through even 60% sucrose. If the protein concentration during reconstitution was substantially under 1 mg/ml, similar results were obtained. Increasing the lipid/protein ratio up to 50:1 led to a progressive increased association of protein with lipid. At a ratio of 50:1, over 90% of protein was found associated with lipid at the top of the gradient (Figure 5.9B). However, the density of the final product was such that it failed to enter even a 3 - 10% sucrose gradient.

As TA-receptor binding is best assayed under conditions of discrete separation of membrane in a gradient, reconstitution conditions were varied to produce a reconstituted fraction with density sufficient to allow separation in either an 8 - 40% or 3 - 15% sucrose gradient. Finally a lipid/protein ration of between 20 and 30:1 and a protein concentration of 1 mg/1 ml were routinely used. Analysis of total nuclear envelope protein reconstituted with lecithin under these conditions is shown in Figure 5.9 C. Routinely, three fractions were obtained: a lipid fraction at the top of the gradient; reconstituted material banding in the gradient (Figure 9, Peak b) and a pellet of unreconstituted protein.

SDS gel electrophoresis of reconstituted material obtained from the gradient, showed similar polypeptide distribution to native nuclear envelope, indicating that selective incorporation of certain proteins is not taking place (Figure 5.8). On occasions, an additional band of lower density was obtained on the gradient (Figure 5.9, Peak A. Only a small amount of protein was in general associated with this band and it was not used for binding studies.

The possibility that the envelope proteins were being enclosed in lecithin liposomes rather than associating directly with the lipid was considered. Brief sonication, designed to disrupt vesicles did not decrease the amount of radioactivity migrating with the reconstituted complex or the density of the complex (Figure 5.10). Only a small amount of ^{125}I labelled insulin could be incorporated into the reconstituted fraction and this could mostly be released by sonication and found at the top of the gradient after centrifugation (Figure 5.10), whereas sonication had no effect on the association of envelope protein with lipid and did not alter the density of the reconstituted material (Figure 5.10). The failure of a nonmembrane protein such as insulin to become significantly incorporated in the reconstituted fraction is good evidence that the extensive association of nuclear envelope proteins with the lipid after reconstitution is due to the preference of these proteins for a hydrophobic lipid environment, rather than nonspecific entrapment in liposomes.

To confirm this result, the reconstituted complex was radioiodinated using Sepharose bound lactoperoxidase as a generator of radicles (see 7.3.9). Proteins entrapped in liposomes rather than associated with a bilayer would not be labelled by this procedure as the short-lived radicles cannot cross the bilayer but require the substrate to be close to the active site of the lactoperoxidase. After labelling, the membrane was subjected to SDS gel electrophoresis and autoradiographed. Extensive labelling of polypeptides can be seen (Figure 5.11). Although these results cannot rule out nonspecific adsorption of protein to to a reformed lipid bilayer, the envelope proteins are likely to favour their native environment and associate with lipid as found for erythrocyte membranes by Zahler and Weibel (1970).

Reconstitution of total nuclear envelope protein with total envelope phospholipid therefore produces a reconstituted complex with a density much closer to that of the original envelope than that obtained when purified lecithin is used. These results probably indicate the preference of certain envelope polypeptides for particular lipids. Kramer et al., (1972) found preferential binding of sphingomyelin by sheep erythrocyte membrane proteins. The large lipid:protein ratio needed for reconstitution with a single lipid, lecithin, has been observed by a number of authors (Eytan and Kanner, 1978).

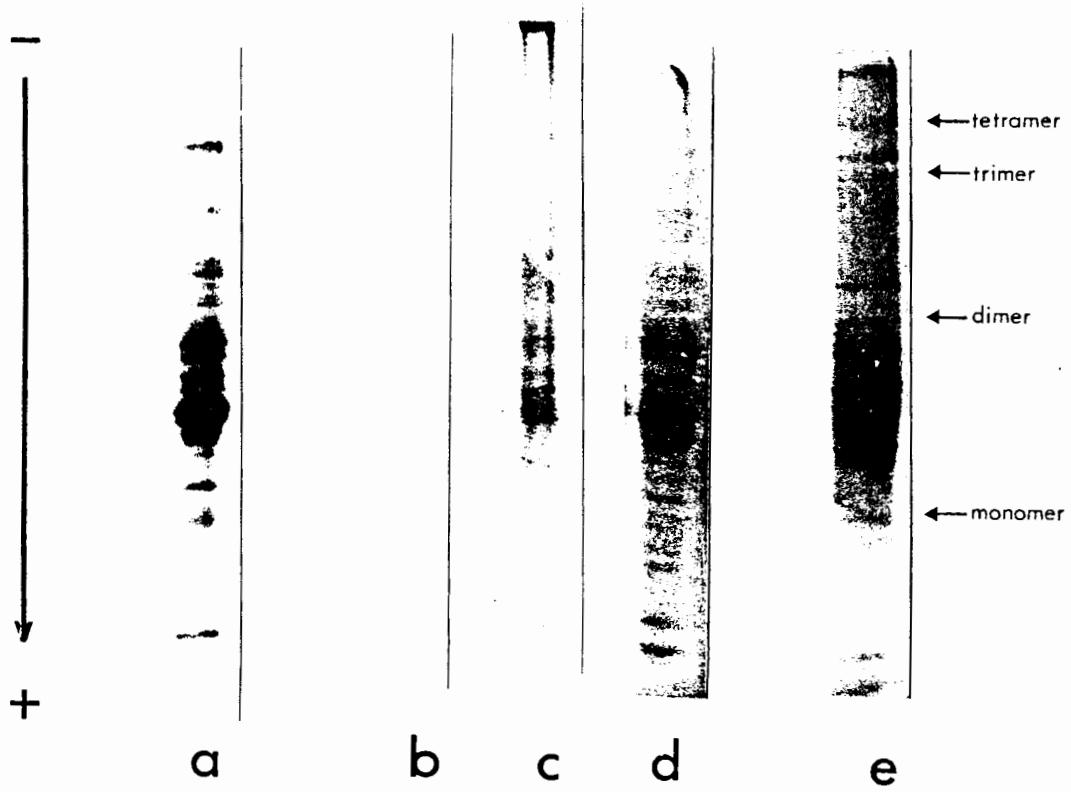

FIGURE 5.8

SDS gel electrophoresis of reconstituted material.
Gels were stained for protein with Coomassie Brilliant Blue.

(a) nuclear envelope

Total envelope protein reconstituted with envelope lipid and fractionated on a sucrose gradient (Figure 5.7).

(b) top of gradient
(c) reconstituted material
(d) pellet

(e) total envelope protein reconstituted with lecithin (Figure 5.9, Peak B)

Arrows indicate molecular weight marker proteins:

monomer = 53 000 daltons (BDH Product No. 44230)

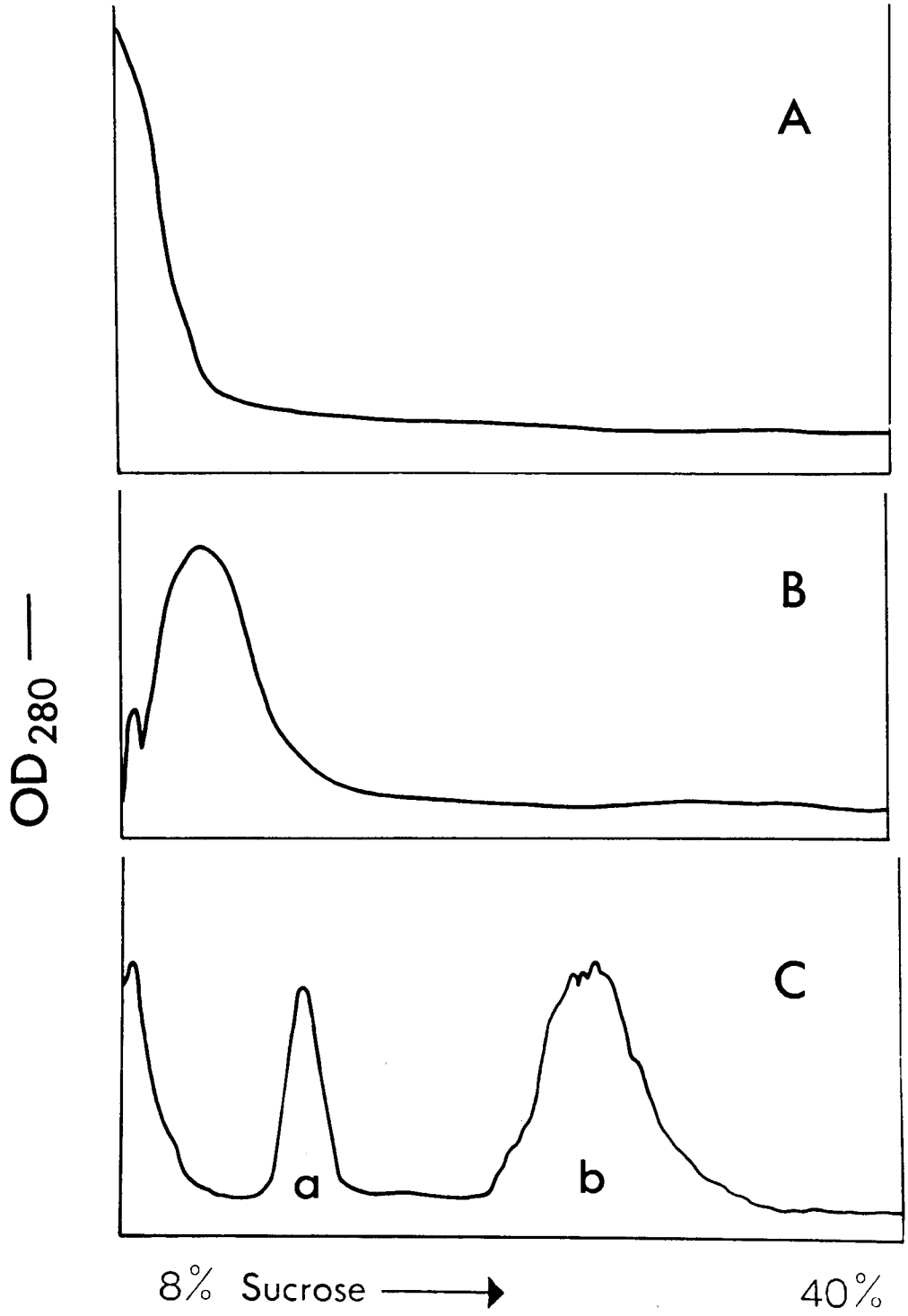

FIGURE 5.9 Reconstitution of lipid free total nuclear envelope protein with lecithin under conditions described in 5.4.2.

Reconstituted membranes were centrifuged for 30 minutes at 100 000 g on an 8 - 40% sucrose gradient.

A. Lecithin/protein ration of 5:1
B. Lecithin/protein ratio of 50:1
C. Lecithin/protein ration of 20:1

Of the two peaks banding in the gradient, the one of lower density, (a), was not always found. Peak (b) represents the predominant product.

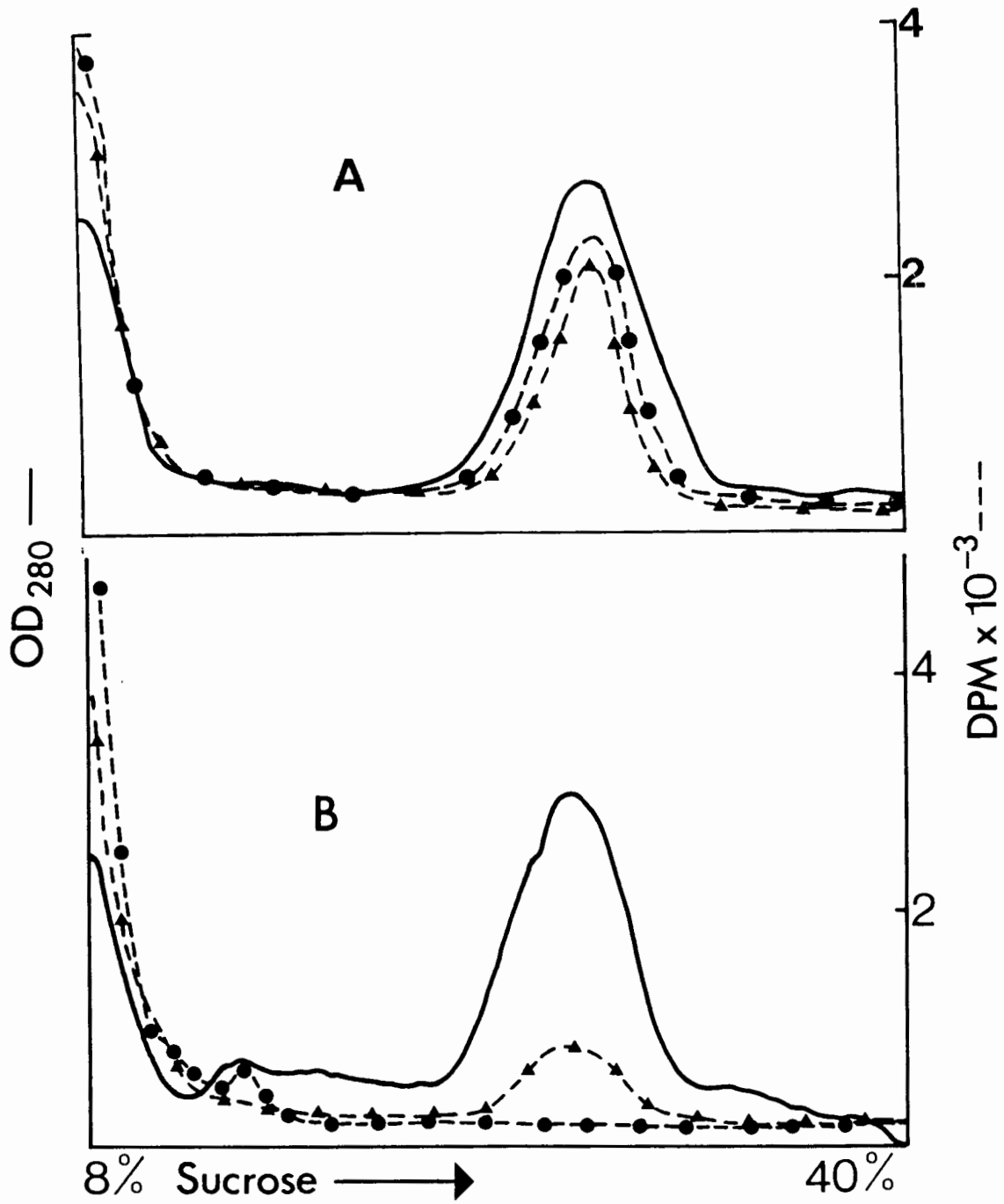

FIGURE 5.10 Reconstitution of lipid free envelope protein with lecithin as described in 5.4.2. The reconstituted material was analysed on an 8 - 40% sucrose gradient.

———— OD_{280}

●—●—● dpm after sonication

▲—▲—▲ dpm prior to sonication

A. Reconstitution using ^{125}I labelled nuclear envelope protein.

B. Reconstitution using unlabelled nuclear envelope protein and 100 µg of ^{125}I labelled insulin.

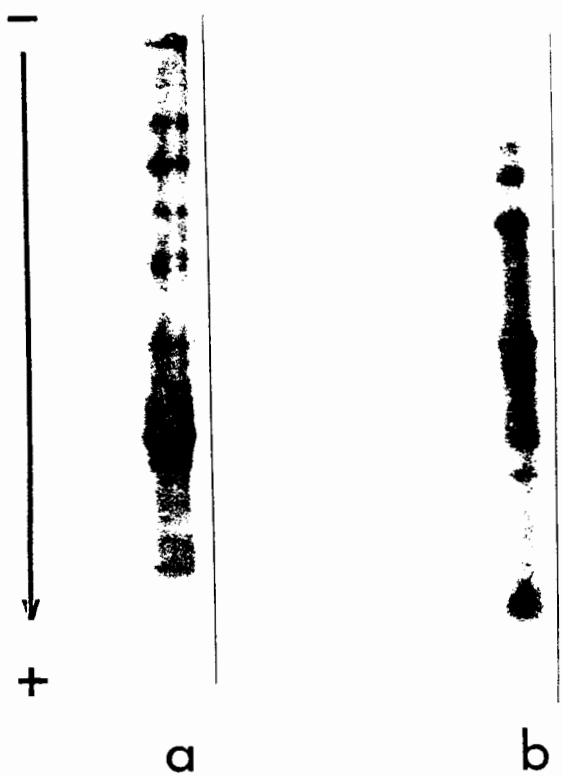

FIGURE 5.11 Autoradiography after SDS gel electrophoresis of

(a) nuclear envelope

(b) reconstituted total nuclear envelope.

Reconstituted total nuclear envelope was collected after sucrose gradient sedimentation and iodinated with ^{125}I as described (7.3.9).

5.5 INTERACTION OF TA-RECEPTOR COMPLEX WITH RECONSTITUTED MATERIAL

Total nuclear envelope protein obtained after fractionation on LH20 (see 5.3) was reconstituted with either total native lipid or lecithin as described (5.4) and fractionated on the appropriate sucrose gradients. The reconstituted fractions were collected by aspiration, pelleted and incubated with TA-receptor complex as described (7.4.1). Samples were again centrifuged on the appropriate gradient and the gradients fractionated and assayed for radioactivity. As controls, envelope lipid and lecithin were taken through the reconstitution procedure without the addition of protein. Because lipid alone could not be well separated on a sucrose gradient, TA-receptor binding was assayed after incubation by pelleting the lipid and extracting bound radioactivity with 0,3 M KCl. Results are expressed in Tables 5.2 and 5.3.

Membranes reconstituted using envelope lipid bound the TA-receptor with a specific activity (dpm bound per mg protein) of about 40% of that of the native envelope. This value is slightly lower than that obtained for whole nuclear envelope reconstituted from chloroethanol (see 5.2). Membranes reconstituted with lecithin alone bound even less TA-receptor. The specific activity of this fraction was 27% that of isolated envelope. Both lecithin and envelope lipid alone failed to bind the TA-receptor, even in the presence of excess lipid (Table 5.3).

These results show that the envelope acceptor for the TA-receptor complex is located in the protein rather than lipid fraction on the envelope. The acceptor survives fractionation on Sephadex LH20 in 2-chloroethanol.

TABLE 5.2

BINDING OF TA-RECEPTOR COMPLEX TO MATERIAL RECONSTITUTED USING TOTAL NUCLEAR ENVELOPE PROTEIN AFTER CHROMATOGRAPHY ON SEPHADEX LH 20

FRACTION	DPM per 100 µg PROTEIN
Envelope protein reconstituted with lecithin	270
Envelope protein reconstituted with envelope lipid	560
Native nuclear envelope	1020

Input radioactivity was 10 000 dpm. 200 µg membrane protein was used per incubation. Incubation conditions are described in 7.4.1.

TABLE 5.3

BINDING OF TA-RECEPTOR COMPLEX TO NUCLEAR ENVELOPE LIPID

FRACTION		DPM IN PELLET
Nuclear envelope (100 µg)		1896
Total envelope lipid	1 mg	953
	2 mg	1061
	3 mg	1083
Blank (no membrane or lipid present)		1027

Results represent an average of three determinations.

The fractions were incubated with 7 000 dpm of TA-receptor complex for 1 hour at +4^{o}C and pelleted by centrifugation for 15 minutes at 30 000g. Pellets were washed twice in TGA buffer and then solubilized in SDS and assayed for radioactivity.

5.6 FRACTIONATION OF NUCLEAR ENVELOPE ON SEPHACRYL S200 SUPERFINE

Though protein and lipid are well separated on Sephadex LH 20, little fractionation of the protein component was achieved. Fractionation of nuclear envelope proteins was attempted using Sephacryl S200 Superfine. This matrix was stable in 90% 2-chloroethanol 0,01 M Na Phosphate, pH 7,5. The column size and elution conditions were the same as those used with Sephadex LH 20. Protein and phospholipid were monitored as before (5.3). Figure 5.12 shows a typical elution profile. Phospholipid was completely separated from protein and some fractionation of the protein component was achieved. The extent of protein fractionation was assessed by subjecting aliquots of the various fractions to SDS gel electrophoresis (Figure 5.12, inset). The high molecular weight proteins, in particular the prominent 170 kilodalton polypeptide was highly enriched in the first peak, and was well separated from the rest of the proteins. Further fractionation was not obtained. The second broad peak contained the remainder of the lower molecular weight proteins.

Fractions 25 - 29 and 34 - 48 (Figure 5.12) were pooled, concentrated under nitrogen at +4^{o}C to a final protein concentration of 1 mg/ml, and reconstituted with lecithin by dialysis against 0,1 M NaCl, 0,01M Na phosphate, pH 7,5, as previously described (5.4.2). The contents of the dialysis bags were layered over 3 - 15% sucrose gradient and centrifuged for 2 hours at 38 krpm in a SW 40 Ti rotor. The reconstituted fractions were recovered from the gradients and incubated with TA-receptor complex, and again centrifuged on a 3 - 15% gradient. The gradients were fractionated and assayed for radioactivity (Figure 5.13). Reconstituted fractions containing the high molecular weight proteins did not bind TA-receptor complex (Figure 5.13a). Membranes reconstituted using protein from fractions 34 - 48 (Figure 5.12) did bind TA-receptor complex (Figure 5.13b). Bound radioactivity could be extracted from the membranes with 0,3 M KCl. Free steroid failed to bind either of the reconstituted membranes.

Prolonged exposure to 2-chloroethanol led to a slow breakdown of the Sephacryl S200 Superfine and a change in chromatographic properties of the column resulting in no fractionation of protein being obtained. Attempts to obtain more fractionated envelope protein from this potentially useful matrix in order to quantitate TA-receptor binding to reconstituted material were abandoned when it was found that the properties of the Sephacryl S200 in 2-chloroethanol varied from batch to batch of Sephacryl

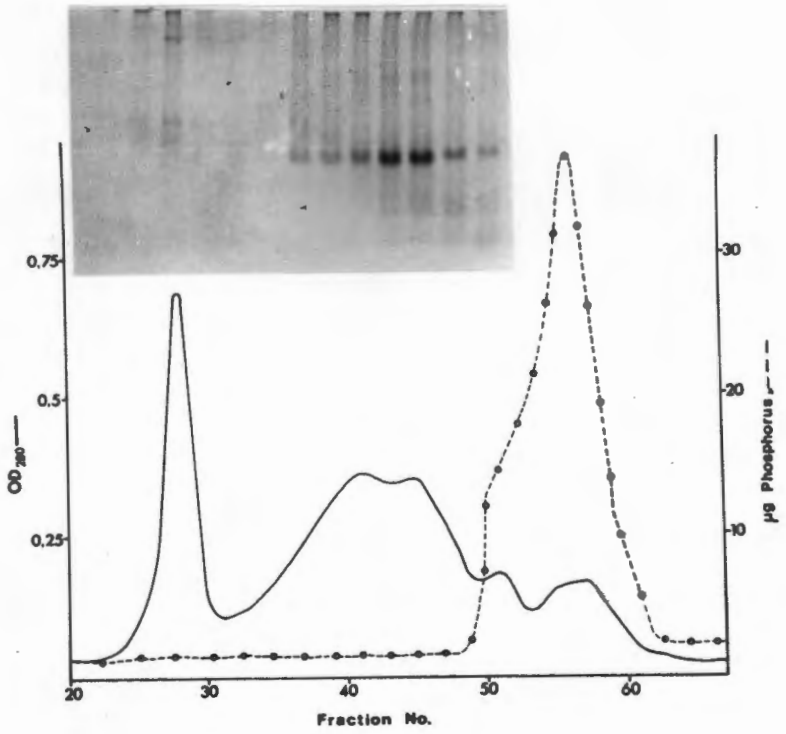

FIGURE 5.12 Fractionation of nuclear envelope on Sephacryl S200 Superfine. Column size and elution conditions were identical to those employed in Figure 5.5.

Inset: SDS gel electrophoresis of various fractions under conditions described in 7.3.1.

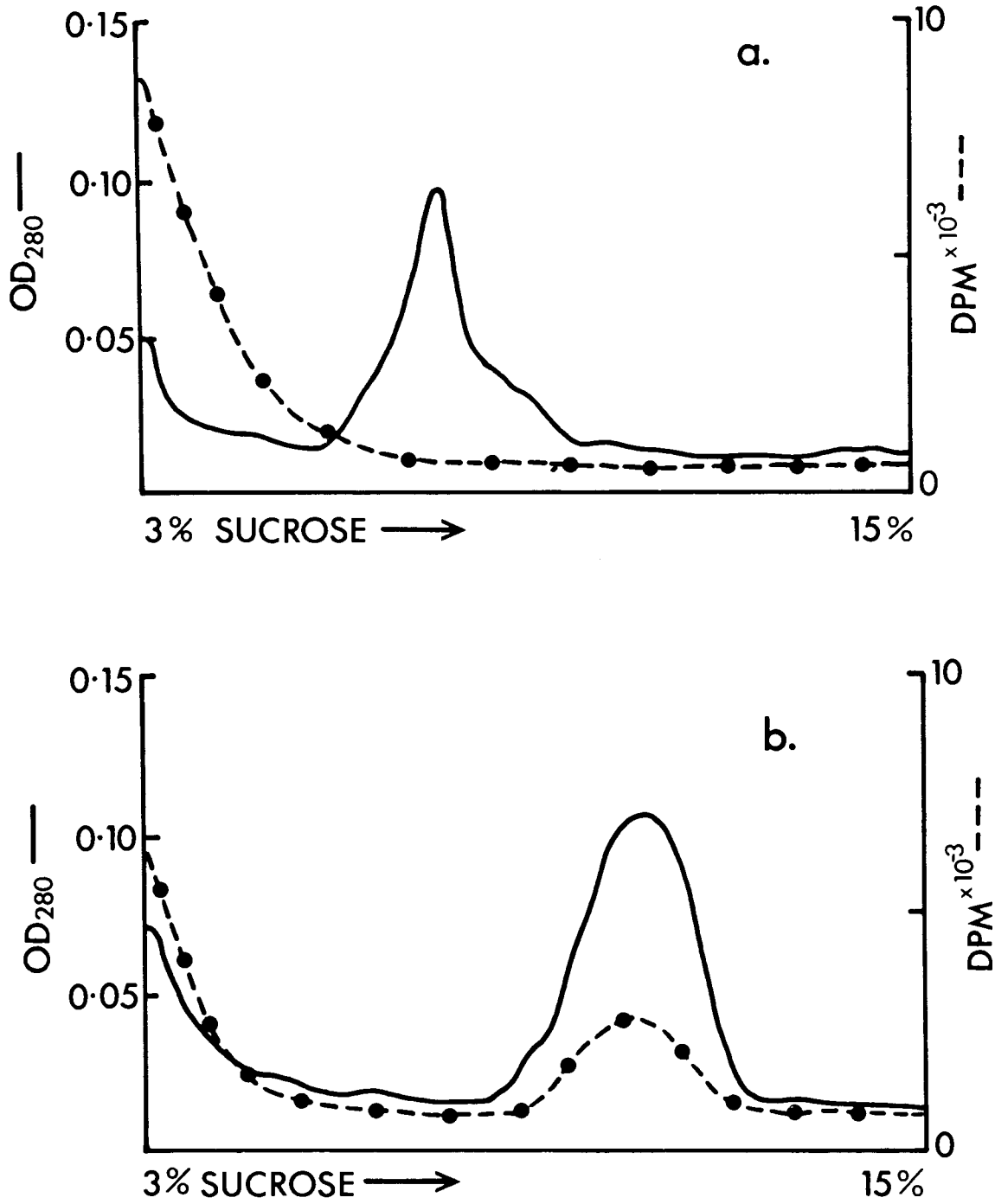

FIGURE 5.13 Sedimentation diagrams of reconstituted material after incubation with TA-receptor complex.

(a) Material reconstituted using protein from fractions 25 - 29 (Figure 5.12) after chromatography of the nuclear envelope on Sephacryl S200 Superfine.

(b) Material reconstituted using protein from fractions 34 - 48 (Figure 5.12).

purchased. One batch developed strong ion exchange properties when exposed to 2-chloroethanol, to the extent that a large proportion of protein remained bound to the column. A further batch was even less stable to chloroethanol and all protein eluted with phospholipid in the inner column, indicating possible breakdown of the column. The suitability of sililated controlled pore glass as a fractionating matrix was also investigated and although stable to chloroethanol, no fractionation was obtained with any of the available pore sizes.

Although work with Sephacryl S200 Superfine was terminated due to instability of the matrix, the preliminary results presented above indicate that the group of proteins thought to include the pore complex polypeptides also contain the binding site for the TA-receptor complex. Further fractionation of envelope proteins by gel exclusion chromatography in 2-chloroethanol was ruled out due to lack of suitable matrices stable in this solvent.

To further investigate the location of the TA-receptor binding site in the envelope it was decided to attempt selective solubilization of envelope proteins with a suitable detergent. The nuclear pore complex and underlying fibrous lamina are known to be insoluble even in high concentrations of nonionic detergents (Aaronson and Blobel, 1974).

5.7 DETERGENT EXTRACTION OF THE NUCLEAR ENVELOPE

5.7.1 Extraction with Triton X-100 and Lithium diiodosalycilate

Two detergents were initially chosen for attempted selective extraction of polypeptides from the envelope. These were Triton X-100 and lithium diiodosalycilate (LIS). Triton X-100 is a nonionic detergent which disrupts the nuclear envelope mainly by extraction of the lipid component. The inefficiency of Triton X-100 as a solubilizing agent for nuclear envelope proteins is referred to in detail elsewhere in this thesis (1.3.1.2). A large number of polypeptides, including the pore complex, are not extracted by this detergent (Aaronson and Blobel, 1974). It is probable that the only proteins which are solubilized by low concentrations of Triton X-100 are loosely bound peripheral proteins. Lithium diiodosalycilate, a dialysable detergent, has been observed by Marchesi and associates (Marchesi and Andrews,1971) to offer a unique utility in the solubilization of membrane glycoproteins; in their case, band three of erythrocyte glycophorin. Either of these detergents might thus be potentially useful in determining whether an integral or peripheral envelope protein fraction is responsible for the binding of TA-receptor complex to the nuclear envelope.

Nuclear envelope buffered in 0,01 M Tris, pH 7,5 was extracted with either Triton X-100 or LIS in the concentration range 0,01 - 1%. Insoluble material was pelleted by centrifugation and both pellet and supernatant analysed by SDS gel electrophoresis. Detergents were removed from the samples by precipitation of protein with 10 volumes of acetone prior to electrophoresis in the case of Triton X-100 or by dialysis in the case of LIS. Results are shown in Figure 5.14. Increasing concentrations of both Triton X-100 and LIS can be seen to extract progressively larger amounts of polypeptides from the envelope (Table 5.4). Extraction with either 0.1% Triton X-100 or 0.1% LIS gives an enrichment in the bands running on SDS gels, directly behind the prominent triplet which has been proposed to contain the pore complex polypeptides. Increasing concentrations of either detergent failed to extract a number of polypeptides, including the prominent high molecular weight band and a prominent component of the triplet. 1% LIS solubilized over 60% of envelope protein whereas lower concentrations extracted only between 2 and 6% (Table 5.4).

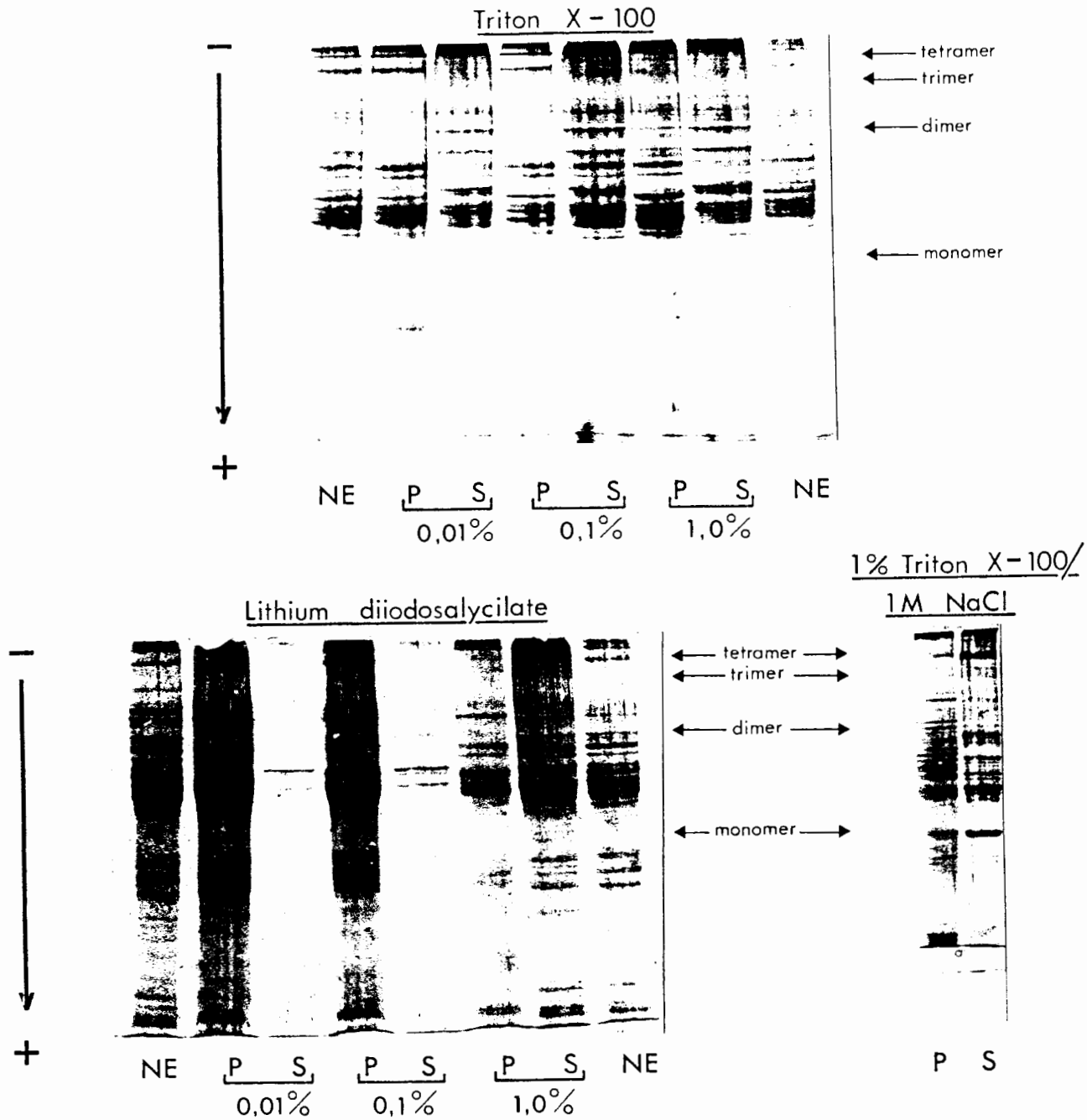

FIGURE 5.14 Extraction of nuclear envelopes with varying concentrations of Triton X-100 and Lithium diiodosalycilate. Extracted protein was precipitated with acetone, dissolved in sample application buffer and subjected to SDS electrophoresis on 10% polyacrylamide gels.

S = supernatant

P = pellet

NE = nuclear envelope (run as standard)

Arrows indicate molecular weight marker proteins:
monomer = 53 000 daltons (BDH Product No. 44230)

TABLE 5.4

EXTRACTION OF NUCLEAR ENVELOPE WITH DETERGENTS

DETERGENT (percent)		SOLUBILIZED PROTEIN (percent)
Triton X-100	0,01	5,3
	0,10	10,0
	1,00	21,5
LIS	0,01	2,4
	0,10	6,8
	1,00	61,2
1% Triton X-100, 1 M NaCl		82,0

Aliquots of envelope containing 200 µg protein were used for each extraction. Solubilized protein was precipitated with TCA and assayed by the Folin Lowry procedure.

The envelope was also extracted with 1 M NaCl, 1% Triton X-100 and insoluble material pelleted by centrifugation for 1 hour in a 75 Ti rotor at 50 000 rpm. Krohne et al.,(1981) have observed that simultaneous treatment of nuclear envelopes with 1 M NaCl and 1% Triton X-100 produces an insoluble fraction highly enriched in pore complexes. Figure 5.14 shows that the insoluble fraction is highly enriched in a few bands, but that a large number of minor bands are also present. About 80% of total envelope protein was solubilized by this procedure (Table 5.4).

5.7.2 Reconstitution with Nuclear Envelope Lipid

Detailed reconstitution procedure followed is outlined in Methods (7.4.6). Both detergent-soluble and -insoluble fractions of the envelope were reconstituted with total envelope lipid from chloroethanol by dialysis against 0,1 M NaCl, 0,01 M Na phosphate, pH 7,5. The contents of the dialysis bags were removed and applied to an 8 - 40% sucrose gradient and centrifuged for 2 hours at 38 krpm in a SW 40 Ti rotor. Analysis of two gradients is presented in Figure 5.15. Both the detergent-soluble and detergent-insoluble fractions could be successfully reconstituted with native envelope lipid. As is evident from Figure 5.15, the densities of the reconstituted complexes varied with the protein fraction used for reconstitution. Complexes reconstituted using detergent extracts had generally lower densities than those reconstituted with detergent-insoluble proteins, due to the fact that detergent extracts usually contained less protein than the non-soluble fractions. SDS electrophoresis once again confirmed the presence of protein in the reconstituted membranes (Figure 5.16). Percent incorporation of protein is given in Table 5.5.

Some problems were experienced in reconstituting proteins extracted with 1% Triton X-100 and 1 M NaCl/1% Triton X-100 due to the difficulty of Triton removal by dialysis at this concentration. Excessively long dialysis times were needed before reformed material was obtained. This problem was solved by addition of a 10-fold excess of lipid prior to reconstitution. Resultant reconstituted material had a ligher lipid/protein ration, but overnight dialysis removed sufficient Triton X-100 to allow reconstitution to take place and protein to associate with lipid.

TABLE 5.5

INCORPORATION OF DETERGENT SOLUBLE AND INSOLUBLE ENVELOPE FRACTIONS INTO MEMBRANES RECONSTITUTED USING ENVELOPE LIPID

FRACTION		PERCENT INCORPORATION OF PROTEIN*
0,1% LIS	soluble	83
0,1% LIS	insoluble	92
0,1% Triton X-100	soluble	86
0,1% Triton X-100	insoluble	88
1% Triton X-100 1 M NaCl	soluble	63
1% Triton X-100 1 M NaCl	insoluble	72
Nuclear protein matrix		42

*This represents the fraction of protein found associated with lipid after reconstitution as described in 5.7.2, and sucrose gradient centrifugation, as a fraction of total protein used for reconstitution.

TABLE 5.6

BINDING OF TA-RECEPTOR COMPLEX TO RECONSTITUTED MEMBRANES

FRACTION	DPM BOUND/100 µg PROTEIN
Native nuclear envelope	806
*Total reconstituted nuclear envelope	528
0,1% LIS supernatant 0,1% LIS pellet	70 320
0,1% Triton X-100 supernatant 0,1% Triton X-100 pellet	235 135
1% Triton X-100/1 M NaCl supernatant 1% Triton X-100/1 M NaCl pellet	215 35
Reconstituted nuclear protein matrix	95
Blank (TA-receptor complex alone)	280

Results represent the average of four determinations.

Incubation conditions and assay of bound radioactivity are described in 7.4.7. Each incubation contained 7 000 dpm of TA-receptor complex. Results represent values obtained after blank subtraction.

*Total reconstituted nuclear envelope was obtained by solubilization of nuclear envelope in 2-chloroethanol:0,05 M Na phosphate, pH 7,5 followed by immediate reconstitution by dialysis against 0,1 M NaCl, 0,01 M Na phosphate pH 7,5.

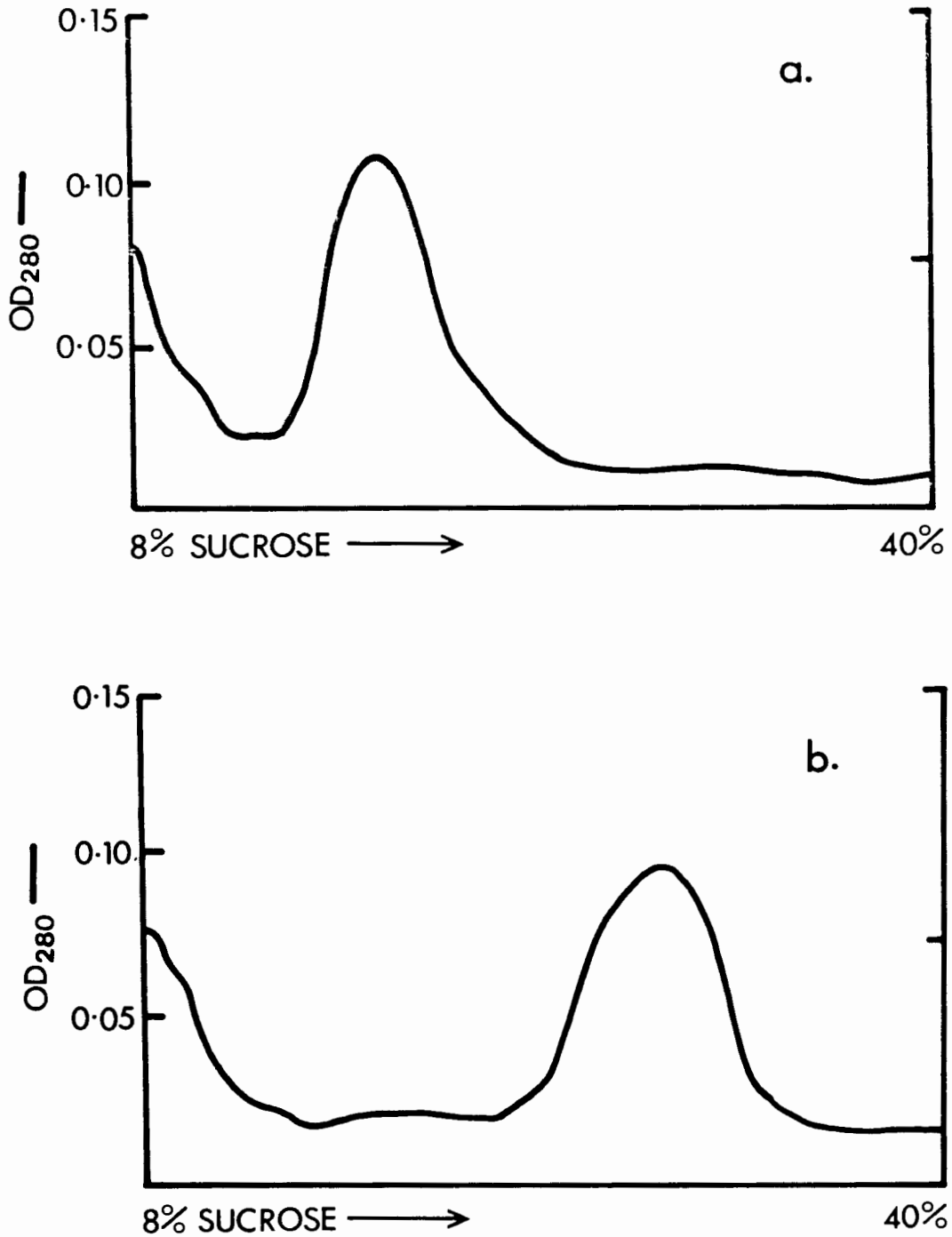

FIGURE 5.15 Sedimentation diagrams of reconstituted material.

(a) Material reconstituted using 0,1% Triton X-100 extracts of nuclear envelope.

(b) Material reconstituted using 0,1% Triton X-100 insoluble residue of nuclear envelope.

The insoluble fraction produced after extraction with 1 M NaCl/1% Triton X-100 was not as readily soluble in 2-chloroethanol as total nuclear envelope and a maximum of 80% of the pellet was solubilized after five minutes exposure to 2-chloroethanol. This fraction is enriched in pore complex and lamina components (Aaronson and Blobel, 1975) and it was not certain whether these proteins would reassociate with lipid. The nature of the association of the lamina with the inner nuclear membrane is unknown and from electron microscopic evidence, the interaction of pore complex material with inner and outer membranes in unclear. However up to 70% of this fraction solubilized by 2-chloroethanol was able to reassociate with envelope lipid on reconstitution.

5.7.3 Interaction of Reconstituted Complexes with TA-Receptor Complex

Binding of TA-receptor complex to material reconstituted from detergent extractable nuclear envelope components was not determined under conditions of sucrose gradient centrifugation. Assaying for binding in a gradient was made difficult by the fact that reconstitution of a single sample often produced reconstituted material with a range of densities and a consequent spread of particles in a gradient rather than a narrow discrete band (Figure 5.15). TA-receptor binding was therefore assayed after incubation by pelleting of the complexes and extraction of radioactivity with 0,3 M NaCl. Nonspecific pelleting of radioactivity was regularly monitored by inclusion of blanks from which reconstituted material was omitted. All incubations were performed using TA-receptor complex from a single preparation and hence identical specific activity. Input of radioactivity was 7 000 dpm (0,5 p mole). Reconstituted material was determined as protein and binding expressed as dpm per 100 µg protein. Results are given in Table 5.6 and represent the average of four determinations.

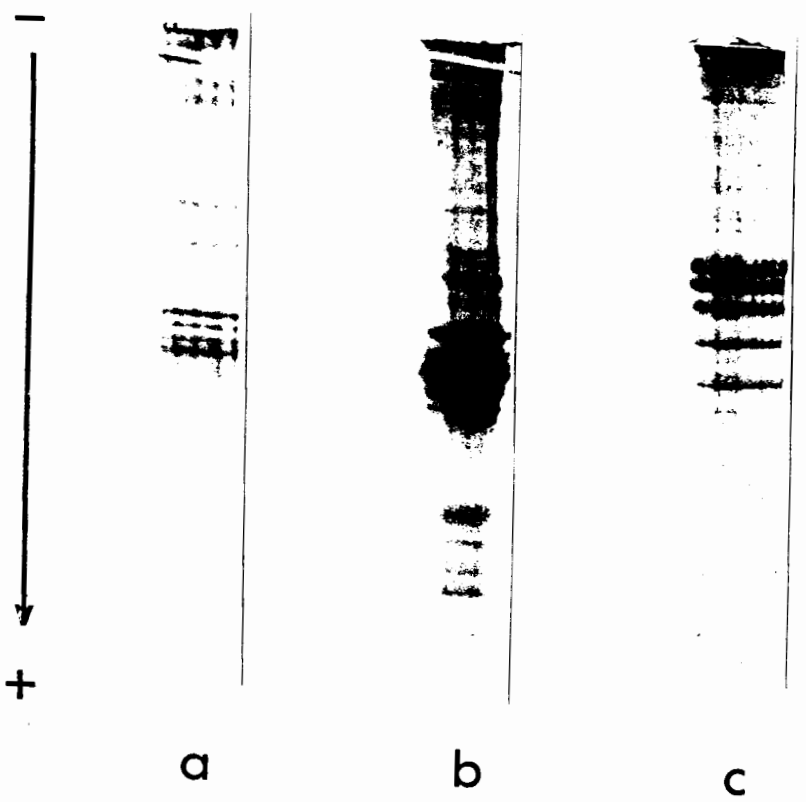

FIGURE 5.16 SDS gel electrophoresis of reconstituted fractions. Reconstitution was performed using protein fraction from

(a) 0,1% Triton X-100 soluble envelope extract

(b) 0,1% Triton X-100 insoluble envelope fraction

(c) nuclear protein matrix

Total reconstituted nuclear envelope i.e. unextracted nuclear envelope, solubilized in 2-chloroethanol and reconstituted by dialysis against aqueous buffer, bound 60% of the radioactivity bound by native nuclear envelope (see 5.2). Thus during reconstitution, 40% of binding activity is lost. 0.1% Lithium diiodosalycilate extracted only a small amount of binding activity - over 80% remained associated with the insoluble fraction. However, when the envelope was extracted with 0,1% Triton X-100, 65% of the TA-receptor binding activity was found associated with the detergent soluble fraction and only 35% with the pellet. Simultaneous extraction of the envelope with 1% Triton X-100 and 1 M NaCl left an insoluble fraction with a TA-receptor binding activity of 15% of that in the soluble fraction. At this detergent concentration, the combined binding activity in soluble and insoluble fractions was only 30% of that of unextracted nuclear envelope, indicating that 70% of the TA-receptor binding sites are lost during extraction and reconstitution. However, this means that 30% of binding sites survive detergent extraction, solubilization in 2-chloroethanol and reconstitution with lipid.

Both soluble and insoluble fractions produced by extraction of the envelope with 1.0% LIS failed to bind TA-receptor complex after reconstitution. Envelope proteins solubilized by 1.0% LIS did not reconstitute well with lipid. A large proportion of protein failed to associate with lipid and aggregated to produce a pellet after gradient centrifugation. It is probable that the acceptor was solubilized by 1.0% LIS but showed no binding of TA-receptor complex, due to the unsuccessful reconstitution of the fraction with envelope lipid.

These results indicate that the nuclear envelope acceptor sites for TA-receptor complex belong to that fraction of envelope proteins which is extractable with the nonionic detergent Triton X-100. The pore complex and lamina fractions of the envelope which are insoluble in Triton X-100 can therefore be excluded as sources of TA-receptor binding. Furthermore, results obtained from fractionation of the envelope on Sephacryl S 200 Superfine in 2-chloroethanol (5.6) indicate that polypeptides with a molecular weight higher than 150 kilodaltons are also not involved in the binding. The binding site thus probably belongs to more loosely bound peripheral envelope proteins which would be readily solubilized along with envelope lipid by a nonionic detergent such as Triton X-100.

5.7.4 Reconstitution of the Nuclear Protein Matrix with Nuclear Envelope Lipid

Previous results have shown that 1. the nuclear protein matrix contains a large number of polypeptides which derive from the envelope (4.7.3) and 2. the nuclear protein matrix does not bind TA-receptor complex. From these results it might be assumed that the nuclear envelope acceptor for TA-receptor complex was extracted by one of the steps used to generate the matrix. If, however, the envelope acceptor depended on the presence of lipid for binding activity then any acceptor present in the matrix would be inactive as the matrix contains virtually no lipid.

An attempt was therefore made to reconstitute those envelope proteins present in the matrix with envelope lipid and check for TA-receptor binding activity. The reconstitution procedure employed for the matrix was identical to that used for detergent insoluble fractions of the envelope (5.7.2). Between 40 and 50% of matrix protein could be incorporated into the reconstituted complex (Table 5.5). SDS gel electrophoresis of the reconstituted protein fraction did not reveal selective incorporation of matrix proteins into the bilayer although quantitative differences in some bands were evident (Figure 5.16). The fact that a large proportion of matrix protein is able to be incorporated into a reconstituted complex supports earlier results which indicate that envelope derived proteins are major matrix components (see 4.7.5).

The reconstituted fractions were incubated with TA-receptor complex and assayed for bound radioactivity as previously described (5.7.3). The results are presented in Table 5.6. The reconstituted matrix does bind TA-receptor complex, albeit with a specific activity of between 10 and 15% that of native nuclear envelope. It binds four fold more TA-receptor per mg protein than the unreconstituted matrix (4.7.4). Thus a small proportion of acceptor sites survive extraction with 2 M NaCl and 1% Triton X-100 which are employed to generate the matrix and regain their steroid receptor binding ability when reconstituted into a lipid bilayer.

5.8 DISCUSSION

Investigations into the role and function of membrane bound proteins are hampered by the lack of routine methods of fractionation of these proteins and reconstitution into the bilayers. Most integral membrane proteins are likely to depend on their specific association with the hydrophobic environment of the membrane lipid bilayer for their enzyme, receptor or transport properties. As integral membrane proteins are inserted into the bilayer during protein synthesis it is not clear how these proteins will spontaneously reassociate with lipid during reconstitution, if at all.

This is the first reported work on reconstitution of nuclear envelope protein and lipid. Although the structure of the reconstituted material could not be visualized due to the nonavailability of electron microscopic techniques, the density and composition of the reconstituted product indicated reassociation of lipid with protein. Based on the results of Zahler and Weibel (1970) it is likely that at least a proportion of the reconstituted material reforms as a bilayer. Reconstitution of total nuclear envelope solubilized in chloroethanol gave a product of similar density to native envelope. The reconstituted envelope bound TA-receptor complex with a specific activity half of that of the native envelope. This evidence indicates reconstitution of a structure with properties similar to native envelope.

Results presented here show that the acceptor site for cytoplasmic TA-receptor complex belongs to that fraction of envelope protein extractable with low concentrations of the nonionic detergent Triton X-100. This result was somewhat surprising, as the pore complex which is non-extractable with Triton X-100, was clearly a strong candidate for the localization of the binding site. One of the problems encountered during this study was the fairly high loss of acceptor activity during solubilization and reconstitution of envelope proteins and hence the need to use larger amounts of reconstituted material to be able to obtain above background binding of TA-receptor complex. This problem and the fact that a TA-receptor complex of much higher specific activity could not be isolated using the isolation protocol outlined in Part 2, prevented the generation of reliable binding data for possible Scatchard analysis. However the fact that any solubilized acceptors at all were able to regain binding activity after spontaneous reassociation with lipid during dialysis is perhaps remarkable in itself.

Although these results indicate that the envelope binding component for TA-receptor complex is a detergent extractable membrane protein, they do not throw any light on the precise location of the binding site in the envelope. Does the TA-receptor complex bind to the inner or outer nuclear membrane? Are the binding sites located near the nuclear pores? The answer to these questions awaits improved methods of fractionation of envelope components or techniques enabling electron microscopic visualization of the envelope-bound TA-receptor complex.

PART 6

CONCLUSION

The experiments reported in this thesis show that the nuclear envelope binds activated cytoplasmic TA-receptor complex with a specific activity ten times higher than the plasma membrane and more than three times higher than the two endoplasmic types of membranes (Table 4.3). The binding constants show two saturable high affinity binding sites and 18 000 binding sites per nucleus. The number of binding sites is similar to those found by other authors (Beato et al., 1974). Lack of binding to the envelope exhibited by the free steroid in the concentration range tested demonstrated that the protein component of the receptor complex mediates binding. Saturable binding to chromatin was not observed in the concentration range tested, due probably to a vast excess of polyanionic DNA sites in the chromatin. The lectins Con A and LCH did not compete with the TA-receptor complex for binding to the envelope, ruling out the possible involvement of mannose and glucose, prominent components of envelope glycoproteins in acceptor activity.

Results obtained from reconstitution experiments confirm that the TA-receptor complex binds to a protein rather than lipid component of the envelope. This component is extractable by concentrations of nonionic detergent which do not extract the pore complex or lamina components of the envelope and is therefore probably a loosely bound membrane protein. The implications of this finding in terms of functional significance of the association of the TA-receptor complex with the envelope are not readily evident. The interaction of the glucocorticoid receptor complex with a non-integral nuclear envelope protein may simply be one linked to the transport of the complex into the nucleus, presumably through a pore. The pore complex components, which are not solubilized by Triton X-100, did not bind the TA-receptor complex after reconstitution with lipid. However the pore complex components have not yet been fully characterized and the possibility that loosely bound membrane proteins are associated with the pore complex cannot be excluded. The nuclear transportation of the cytoplasmic receptor-bound steroid appears to result from

inherent properties of the receptor and the temperature dependent activation of the TA-receptor complex might allow association with specific acceptors on the envelope. Nenci et al., (1980) who observed receptor bound estradiol associated with the pore complexes also noted the unusually high number of pores in estradiol-treated cells and suggested that interaction of the steroid hormone-receptor complex with the envelope might even induce pore formation.

Current evidence indicates that steroid hormone-receptor complexes enter the nucleus from the cytoplasm (Schaltman and Pongs, 1982) and that receptors isolated from cytoplasm and nucleus are thus identical. If this were not the case, the envelope could clearly mediate in the transfer of steroid from cytoplasmic to nuclear receptor. Despite the fact that glucocorticoid receptor complexes appear to enter the nucleus, part of the glucocorticoid response might be mediated at the envelope level in a manner similar to the way in which the plasma membrane mediates protein and peptide hormone signals from outside the cell. The protein component of the steroid hormone-receptor complex could elicit a similar type of response at the nuclear membrane. The extensive association of DNA with the inner nuclear membrane raises the possibility that certain genes may be anchored to distinct areas of the envelope. Receptors in these areas of the envelope could then be envisaged to orientate cytoplasmic signals destined for those genes.

Few studies on the interaction of steroid hormones or steroid hormone-receptor complexes with the nuclear envelope have been done. Work by Jackson and Chalkley (1974a) and Lefebvre and Morante (1982) showed respective binding of free estradiol-17-β and dihydrotestosterone to nuclear envelopes. I have been unable to show significant binding of non receptor-bound steroid to the envelope in the concentration range used. Jackson and Chalkley (1974a) demonstrated high and low affinity binding sites in the nuclear envelope of bovine endometrium for 17-β -estradiol. The high affinity site was not observed in thymus or immature calf uterine tissue. The low affinity sites were not saturable and were shown to represent nonspecific association of the steroid with the membrane. Lefebvre and Novosad (1980) and Lefebvre and Morante (1982) investigated binding of dihydrotestosterone to nuclear envelopes and also found a large proportion of observed binding to be

nonspecific. The above studies were all performed with free steroid rather than steroid hormone-receptor complex. Nenci et al., (1980), using immunofluorescent techniques, observed receptor-bound estradiol on the nuclear envelope, particularly in the vicinity of the nuclear pore complexes.

Despite many attempts to localize the nuclear binding site for cytoplasmic steroid-hormone receptor complexes, the chemical characterization of nuclear acceptors still awaits elucidation. It has been widely accepted that the acceptor sites belong to that nuclear subfraction designated chromatin and early studies favoured DNA as the binding component (Rousseau et al., 1975). Activation of the cytoplasmic steroid hormone-receptor complex was found to be necessary for binding to DNA and non steroid-bound receptors failed to bind DNA. It was thus easy to postulate that the receptor complex recognized a specific nucleotide sequence in the DNA. However more detailed studies revealed a number of differences in the binding of receptor complexes to nuclei or DNA (see 1.3.1). In particular, binding to DNA was strongly inhibited at elevated ionic strengths (100 mM) (Rousseau et al., 1975) and activated steroid hormone-receptor complexes failed to distinguish between DNA from a variety of eukaryotes and prokaryotes (Simons et al., 1976). The activated complex also bound polyanions such as phosphocellulose (Atger and Milgrom, 1976).

Another form of binding has been postulated for the progesterone receptor-complex which exists as a dimer composed of two subunits which both bind steroid. It has been proposed that one subunit binds to a specific chromatin acceptor protein (presumably a nonhistone protein) and the second to the adjacent DNA, thereby altering transcription in some unknown way (Buller et al., 1976). In a recent review, Spelsberg (1982) supports this model which can account for nonspecific binding of the progesterone receptor-complex to DNA by allowing the protein binding component to provide site specificity. Spelsberg has, however, been unable to show positive binding of the progesterone receptor-complex to protein, as his proposed protein acceptor fraction must be reannealed to DNA to achieve binding activity (Spelsberg et al., 1976).

However, a number of recent publications have again raised the question of whether steroid hormone-receptors recognize specific DNA sequences (for review, see Tata, 1982). Mulvihill et al., (1982) using the DNA-binding subunit of the chick oviduct progesterone-receptor complex found a number of cloned double stranded fragments from genes regulated by progesterone (amongst them ovalbumin) which competed with calf thymus DNA for the progesterone-receptor complex. Hughes et al., (1981) found no DNA sequence specificity for the binding of the progesterone receptor to any region of the ovalbumin gene and found preferential receptor binding to single rather than double stranded DNA. Compton et al., (1983) found preferential binding of oviduct progesterone receptor A to A - T rich DNA fragments flanking the 5' end of the ovalbumin gene.

Using activated glucocorticoid-receptor complex, Payvar et al., (1981) found selective binding "in vitro" to cloned fragments of mouse mammary tumor virus (MMTV) DNA. MMTV DNA is strongly stimulated to transcribe by glucocorticoids after the introduction into the genome of a receptor-containing cell. Govindan et al., (1982), also using MMTV DNA, found specific binding of purified glucocorticoid hormone-receptor complex to the long terminal repeat (LTR) sequences of the DNA. Inititation of transcription occurs within the LTR region. Payvar et al., (1981), however, found binding to sites outside of the LTR region as well.

Thus for both progesterone and glucocorticoid receptor complexes, sequence-specific binding of DNA has been shown, although the results differ on the location of the binding sites. All the studies have been performed under conditions well below physiological ionic strength (50 mM NaCl). Only Mulvihill gives data for binding at higher ionic strengths. The relevancy of the binding observed, to the physiological action of steroid hormones remains to be shown.

Further evidence pointing to DNA involvement in the glucocorticoid response comes from Huang et al., (1981) who succeeded in constructing a plasmid containing the p21 gene (without promotor sequence) of Harvey murine sarcoma virus and the long terminal repeat (LTR) DNA of mouse mammary tumor virus inserted 5' upstream from the p21 gene. The inclusion of the MMTV LTR sequence conferred glucocorticoid sensitivity on the p21 gene which is normally not under glucocorticoid control. This result clearly implicates DNA in the glucocorticoid response and supports the results of Govindan et al., (1982) showing receptor binding to LTR DNA. However the LTR region is sufficiently large to code for a protein which might mediate the response.

Using a similar approach, Pavlakis and Hamer (1983) constructed bovine papilloma virus recombinants containing the promotor and presumptive control region of mouse metallothionein-I gene (MT - I) fused to human growth hormone structural sequences. MT - I gene expression is inducible by both heavy metals and by glucocorticoids. The hybrid mRNA produced in mouse cells transformed with the recombinants, was found to be under cadmium but not glucocorticoid control. Glucocorticoids thus regulate the MT - I gene by a mechanism independent of heavy metals and not by simply increasing the concentration of intracellular metal. The MT - I DNA present in the hybrid therefore somehow specifies heavy metal but not glucocorticoid inducibility. The authors speculate that glucocorticoid regulatory sequences of the MT - I gene might possibly lie outside the sequences used in the hybrid. On the other hand, glucocorticoid inducibility might depend on a specific chromatin environment not found in the transformed cells.

Despite renewed interest in sequence specific interaction of steroid hormone-receptor complexes with DNA, the question of the large number of binding sites per nucleus (up to 15 000) remains. Does the receptor complex bind to all DNA polyanionic sites with low affinity and only a few specific sites with high affinity? Do chromatin proteins mediate or modulate the binding? Does the steroid receptor complex act as a helix destabilizing protein as proposed by Hughes et al., (1981)? The work of Huang et al., (1981) probably offers the best possibilities to further evaluate the role of DNA as an acceptor for the receptor complex as they have at least identified a distinct stretch of DNA from mouse mammary tumor virus,which specifies the glucocorticoid response.

Work by Gronemeyer and Pongs (1980) and Schaltman and Pongs (1982), using ecdysterone and the salivary glands of Drosophila melanogaster, suggests that their steroid hormone-receptor complex modulates transcriptional activity directly at the level of the gene. Using immunofluorescent techniques, they were able to show that ecdysterone could be bonded on irradiation to specific hormone controlled puffs of polytene chromosomes. The molecular target of the photoreaction is a 130 000 m.w. polypeptide found in both cytoplasm and nucleus which translocates from cytoplasm to nucleus after binding the steroid. Irradiation did not affect the ability of the complex to translocate to the nucleus although the photo-induced hormone-receptor complex did not readily return to the cytoplasm.

If the cytoplasmic receptor complex does indeed have an intranuclear binding site comprising DNA or protein or both, the possible involvement of the nuclear envelope in the glucocorticoid response can nevertheless not be ruled out. The envelope represents the first point of contact of the steroid hormone-receptor complex with the nucleus and this contact could possibly initiate catalytic responses of the general kind produced by certain peptide hormones at the plasma membrane. This kind of response could lead to a modification of chromatin structure in general or possibly an alteration in a few specific genes. Johnson et al., (1979) have shown that glucocorticoids rapidly induce a major modification of chromatin structure under conditions in which the hormone affects only a small subset of genes. In cultured pituitary cells, they found that dexamethasone induced an increase of 500 000 initiation sites for bacterial polymerase - i.e. ten times more sites than steroid hormone-receptor complexes. In this instance, a site specific, one receptor-one polymerase model is excluded. The authors do not attach much significance to the number of initiation sites, but suggest that the assay detects rather a change in chromatin structure which has no obvious bearing on where the polymerase initiates. This response could be catalytically initiated at the envelope. Subsequent interaction of the steroid hormone-receptor complex with an intranuclear binding site could further mediate the response.

All studies (including this one) on the interaction of glucocorticoid hormone receptor complexes with the nucleus point to the interaction of the complex with one or the other nuclear component which is then proposed to mediate the glucocorticoid response. Many models have been proposed but the details of the biochemical events occurring between interaction of the hormone-receptor complex with the nucleus and the production of specific mRNAs still elude workers in the field. Little definitive information is available to determine the precise way in which steroid hormone-receptors affect chromatin. Part of the problem is a lack of availability of homogenous hormone receptor preparations.

Availability of homogenous glucocorticoid receptor complexes should also allow a more detailed investigation as to whether the complex has for example nuclease, protease or phosphatase activity. Any of these activities could correspond to the primary mechanism of steroid action Enzyme activity cannot be directly linked to the complex while only partially purified preparations are used. The interaction of the complex

with the nuclear envelope may, for example, lead to specific modification of a membrane component. Is the extensive modification of nuclear proteins observed after steroid treatment (Allfrey et al., 1973) involved in mediating the glucocorticoid response or do these modifications occur rather as a result of gene activation?

Recently, increased interest has been shown in the nuclear envelope as a target for peptide hormones which interact with the plasma membrane and are internalized by the cell. Yanker and Shooter (1979) using ^{125}I labelled nerve growth factor (NGF), found low and high affinity sites in the envelope for NGF. Vigneri et al., (1978) and Horvat (1978) have found that insulin binds specifically to the nuclear envelope. Whether these interactions are physiologically significant has not yet been established, but both hormones are known to stimulate transcription. Work on epidermal growth factor has shown that certain hormone-stimulated events do not proceed without internalization.

The binding of the cytoplasmic steroid-hormone receptor complex to the nuclear envelope, established in these experiments together with findings from other laboratories on the specific binding of insulin (Vigneri et al., 1978; Horvat, 1978) and also of neural growth factor (Yanker and Shooter,1979) to the nuclear envelope reveal that the nuclear envelope may be the target for specific cytoplasmic hormonal signals which are known to lead to increased nuclear activity. The nuclear envelope may thus be considered as the first relay station in such cytoplasmic nuclear communications.

PART 7

MATERIALS AND METHODS

7.1 MATERIALS

All radioactive chemicals were obtained from the Radiochemical Centre, Amersham.

Heparin (Sodium salt) and unlabelled triamcinolone acetonide, DNase I, Pancreatic RNase and 2,5 diphenyoxazole were obtained from the Sigma Chemical Company.

All other chemicals and reagents were obtained from Merck (Germany).

7.2 ISOLATION PROCEDURES

7.2.1 Isolation of Nuclei

Nuclei were isolated according to the method of Blobel and Potter (1966).

White female rats were starved overnight and killed by cervical dislocation. The livers were excised, trimmed and homogenized in 3 volumes of 0,25M sucrose in 0,05M Tris HCl pH 7,5; 0,025M KCl; 0,0015M $MgCl_2$ (TKM) in a teflon/glass Potter homogenizer at $+4^{o}C$. The homogenate was filtered through 4 layers of cheese cloth and centrifuged at 600g for 20 minutes. The supernatant was removed and the pellet resuspended in 9 volumes of 2,3M sucrose TKM and centrifuged at 45 000g for 70 minutes. If not used immediately, the nuclei were resuspended in 2,3M sucrose TKM and stored in liquid nitrogen ($-170^{o}C$).

7.2.2 Nuclear Envelope Isolation

Nuclear envelopes were prepared according to the method of Bornens (1977). Nuclei were suspended in 0,25M sucrose, 2mM sodium phosphate pH 7,85 by homogenization in a teflon-Potter homogenizer to a DNA concentration of 250 µg/ml at $4^{o}C$. Heparin was added slowly with stirring to give a DNA:Heparin ratio of 1. The solution was stirred gently for one hour and then centrifuged for one hour at 50 000g. The pellet, designated crude nuclear envelope, was taken up in 0,25M sucrose 2mM sodium phosphate, layered over a 25 - 50% sucrose gradient, and centrifuged for 3,5 hours at 170 000g in a Beckman SW 40 Ti rotor. The gradient was analysed on a ISCO Density Gradient Fractionator, Model 640, using a 280 nm filter Material banding at approximately 37% sucrose (d = 1,18 - 1,20) was diluted five-fold with 2mM sodium phosphate and pelleted by centrifuging at 50 000g for 30 minutes.

This preparation was taken as pure nuclear envelope.

7.2.3 Isolation of Chromatin

Chromatin was prepared according to the method of Bonner et al., (1968). Nuclei from two rat livers were suspended in 50 ml of 0,05M Tris-HCl

pH 8,0 and homogenized at +4°C in a glass/teflon Potter homogenizer. The homogenate was centrifuged at 10 000g for 10 minutes and the pellet resuspended in Tris buffer and centrifuged again. The final pellet was resuspended in a small volume of 0,05M Tris buffer, layered over 1,7M sucrose in 10mM Tris-HCl pH 8,0 and centrifuged for 2 hours at 50 000g. The pellet, designated chromatin, was resuspended in 0,25M sucrose, 2mM phosphate pH 7,5 and stored at -20°C.

7.2.4 Isolation of Nonhistone Proteins for SDS Gel Electrophoresis

Purified chromatin was suspended in 10mM Tris pH 8,0, to a concentration of 0,5mg DNA per ml. 2N H_2SO_4 was added dropwise to a final concentration of 0,4N. The precipitate was pelleted by centrifugation and washed gently with 10mM Tris pH 8,0. The supernatant containing the histones was discarded. The pellet was resuspended in 1% SDS, 50mM Tris pH 8,0 by homogenization and stirred overnight at room temperature. The DNA was removed by centrifugation at 170 000g for 24 hours and the supernatant dialysed against sample application buffer prior to SDS gel electrophoresis.

7.2.5 Extraction of Histones and Nonhistone Proteins from Heparinized Nuclei and Chromatin

The heparin supernatant obtained after removal of nuclear envelopes was made 5mM in Mg^{++}. DNase I was added to a concentration of 100µg/mg DNA and the solution stirred for 6 hours at 4°C. The solution was then dialysed against water and freeze dried. Histones were extracted from the freeze dried material with 0,25N HCl and the remaining nonhistone proteins were solubilized in SDS sample application buffer to a concentration of 5mg/ml. Both histones and nonhistones were then subjected to SDS gel electrophoresis.

7.2.6 Preparation of Plasma Membrane

Plasma membrane was purified essentially as described by Aronson and Touster (1974). White female rats were starved overnight and killed by cervical dislocation. Livers were perfused with 50ml 0,25M sucrose, 5mM Tris pH 8,0, excised, trimmed and homogenized in 3 volumes perfusion buffer in a teflon/glass Potter homogenizer. The homogenate was passed

through 4 layers of cheese cloth and then spun at 1 000g for 10 minutes. The pellet was rehomogenized twice in 0,25M sucrose, 5mM Tris pH 8,0 and repelleted. Supernatants were pooled and pellets saved.

The supernatant was spun at 33 000g for 7,5 minutes. The resultant pellet was rehomogenized twice and the step repeated. Supernatants were pooled and the pellet discarded. The supernatant was spun at 78 000g for 100 minutes. The pellet from this spin was homogenized with 2,5 volumes of 57% sucrose, 5mM Tris pH 8,0. Twelve ml of the homogenate were placed in a SW 25,1 rotor tube and overlayed with 15 ml of 34% sucrose, 5mM Tris pH 8,0. The rest of the volume was made up with 0,25M sucrose, 5mM Tris pH 8,0. Tubes were centrifuged at 100 000g for 3 hours. The plasma membrane was obtained at the 0,25M / 34% sucrose interface.

The nuclear pellets obtained after the first 1 000g spin were processed exactly as the supernatants except that the 78 000g spin was omitted and in the final centrifugation, 37,2% sucrose rather than 34% was used. The plasma membrane was collected at the 37,2% / 0,25M sucrose interface.

7.2.7 Preparation of Rough and Smooth Microsomes

Rough and smooth microsomal fractions were produced essentially as described by Adelman et al., (1974). Rats were killed and livers perfused and homogenized as for plasma membrane preparation except that perfusion was with unbuffered 0,25M sucrose and homogenization was in 2 volumes 1,0M sucrose. The homogenate was strained through 1 layer of cheese cloth and an equal volume of 2,5M sucrose added and mixed well. The homogenate was transferred to 60 Ti-rotor tubes, overlayed with 1M sucrose and spun at 100 000g for 45 minutes. The supernatant was removed and half a volume of water added during homogenization. The homogenate was then spun at 22 000g for 15 minutes. The supernatant was decanted and saved and the pellets suspended in 25ml of 0,5M sucrose and centrifuged for 15 minutes at 20 000g. The supernatant was pooled with the previously saved supernatants and spun for 15 minutes at 20 000g. Supernatants were decanted and stored and the pellets discarded. The supernatant was transferred to centrifuge tubes, underlaid with 4ml 1,5 M sucrose and 1ml of 2,0M sucrose, both in 0,05 M Tris, pH 7,5, 0,025 M KCl,

5mM $MgCl_2$ and spun for 20 hours at 200 000g. The smooth microsomal fraction was obtained at the homogenate supernatant / 1,5M interface and rough microsomal fraction at the 1,5M / 2,0 M sucrose interface. Each fraction was finally purified by centrifugation on a 25 - 50% linear sucrose gradient. Peaks were pooled and pelleted.

7.2.8 Isolation of Lectins

The isolation of both lectins was based on their ability to bind glucose and its derivatives. They may therefore be purified by essentially a single affinity step utilizing Sephadex G100 - a cross linked dextran as the binding matrix.

7.2.8.1 Isolation of Concanvalin A (Con A)

250g jack bean meal was stirred overnight in 1,5 litres 0,15 NaCl at $4^{o}C$. The suspension was filtered through 1 layer of cheese cloth and re-extracted with 0,15M NaCl. Filtrates were combined and centrifuged for one hour at 15 000g. The supernatant was removed and solid ammonium sulphate added to a concentration of 30% with stirring. The solution was brought to pH 7,0 with ammonium hydroxide and allowed to stir overnight. The precipitate was removed by centrifugation and the supernatant dialysed against water and passed over a column of Sephadex G100 (2,5 x 100cm) equilibrated with 1 M NaCl. The column was washed with 1 M NaCl until free of material absorbing at 230nm, and the Con A eluted with 0,1M glucose. The eluted material was dialysed against water and freeze dried.

7.2.8.2 Isolation of Lens Culinaris Hemagglutinin (LCH)

LCH was extracted from finely ground lentil beans by the same procedures used for Con A. Extracted material was passed over a Sephadex G100 column (90 x 2,5cm) and the column washed with 0,05M Tris pH 8,0 until $OD_{230} \leq 0,02$. LCH was eluted with 0,1M glucose, 0,05M Tris pH 8,1.

The eluted material was dialysed against water and freeze dried.

7.2.8.3 Coupling of Fluorescein Isothiocyanate (FITC) to Lectins

The method of Malucci (1976) was followed.

100mg of the lectin were dissolved in 10ml buffer (1,0M NaCl, 0,01M Na phosphate, pH 7,5, 0,25mM $CaCl_2$, 0,25mM $MnCl_2$). The Con A solution was made 0,1M in α-D-methyl mannoside and the LCH solution 0,1M in glucose in order to prevent coupling of FITC to residues in the ligand binding site. Undissolved material was removed by centrifugation and the pH of the solution raised to 9 with 0,1 volume of 0,5M Na carbonate/bicarbonate buffer. 1,5mg of FITC dissolved in 1ml 0,05M Na carbonate was added with stirring. The solution was left overnight at $4^{o}C$ and dialysed against buffer to remove unbound fluorescein. The solution was diluted with buffer to a final protein concentration of 1mg/ml and stored in the freezer at $-20^{o}C$.

The fluorescein-labelled lectins had the following specific activities (mole fluorescein / mole lectin) : Con A 1.45 ; LCH 0,64. The fluorescein concentration was calculated spectrophotometrically using the molar extinction coefficient (489nm) of $1,18 \times 10^{5}$. Decrease of fluorescein absorption of 30% on binding to protein was taken into account in calculating specific activities. The molecular weights for the lectins were taken as : Con A = 55 000, LCH = 55 000.

7.2.9 Isolation of Triamcinolone-acetonide Receptor Complex

The method of Climent et al.(1977) was followed. All operations were carried out at $+4^{o}C$. Principal buffers used were TSS (0,05M Tris pH 7,5; 0,025M KCl; 5mM $MgCl_2$; 1mM EDTA $(Na)_2$; 1mM β-mercaptoethanol;) and TGA (0,01M Tris pH 7,5; 1mM EDTA $(Na)_2$; 1mM β-mercaptoethanol; 0,01M NaCl; 10% glycerol; 0,1% bovine serum albumin). 4 male white rats were killed and their livers perfused with 50ml 0,25M sucrose TSS. Livers were homogenized in 2 volumes 0,25 sucrose in a glass/teflon Potter homogenizer. The homogenate was centrifuged at 300 000g for 60 minutes in a Beckman 60 Ti rotor. The supernatant was removed by aspiration, taking care not to disturb the lipid-like material at the top of the gradient. The supernatant was then incubated with 5×10^{-8}M (1, 2, 4, ^{3}H) triamcinolone acetonide (TA) with a specific activity of 7,0 Ci/nmol for 30 minutes

and passed over two phosphocellulose columns. The dimensions of the two columns were 10 x 2,5cms and 5 x 2,5cms respectively. Unbound radioactivity was collected and incubated at 20°C for 30 minutes, cooled to +4°C, and applied to a third phosphocellulose column (4 x 1,5cm). This column was washed with TGA buffer until the eluant was free of radioactivity. The NaCl concentration was then raised to 0,5M and the receptor eluted from the column. Radioactive fractions were pooled and precipitated overnight with 0,5 volume of saturated ammonium sulphate pH 7,0. After centrifugation at 50 000g for 1 hour, the precipitate was resuspended in about 300 µl of TGA buffer and an aliquot checked for radioactivity. Alternatively the ammonium sulphate step was omitted and the 0,5M NaCl eluate was immediately divided into small aliquots and frozen. It was then dispensed in 10 or 20µl quantities into Eppendorf vials and stored in liquid nitrogen until used.

7.2.10 Isolation of the Nuclear Protein Matrix

The nuclear protein matrix was isolated according to the method of Berezney and Coffey (1974). All manipulations were at +4°C.

Step 1: Nuclei were suspended in 0,2mM $MgCl_2$, 10mM Tris pH 7,5 (TM buffer) to a protein concentration of 2mg/ml. They were then pelleted by centrifugation at 780g for 30 minutes and extracted twice more in the same buffer.

Step 2: The pellet from Step 1 was resuspended in 2M NaCl, 0,2mM $MgCl_2$, 10mM Tris pH 7,5 to a concentration of 4 mg/ml, incubated for 10 minutes and pelleted at 780g for 40 minutes. This step was repeated three times.

Step 3: The pellet from Step 2 was resuspended in TM buffer and Triton X-100 added to a final concentration of 1%. The solution was centrifuged at 780 g for 20 minutes and the pellet washed twice with TM buffer without detergent.

Step 4: The pellet from Step 3 was resuspended in TM buffer to a protein concentration of 2mg/ml and DNase I and pancreatic RNase added to a concentration of 200µg/ml. The solution was incubated for 30 minutes at 22°C and centrifuged at 780 g for 20 minutes. The pellet was washed twice in TM buffer.

All supernatants and washings were saved for analysis. The final pellet constitutes the nuclear protein matrix and was resuspended in TM buffer and frozen in liquid nitrogen until used.

7.2.11 Extraction of Lipid from Nuclear Envelope

Total nuclear envelope lipid was extracted from nuclear envelopes using chloroform/methanol (2:1). Envelopes were homogenized in 20 volumes of chloroform/methanol and centrifuged. The nonaqueous phase was removed and the aqueous fraction and pellet were re-extracted with chloroform/methanol. Nonaqueous fractions were pooled and stored under nitrogen in the dark until used.

7.2.12 Purification of Lecithin

Crude lecithin obtained from Merck was solubilized in chloroform/methanol (2:1) and passed over a column of aluminium oxide equilibrated in the same solvent. The flow through was collected and analysed by thin layer chromatography on Silica gel H (see 7.3.12). The plate was dried and stained with iodine. Only one spot, corresponding to lecithin, was obtained. Lecithin was stored as a 10 mg/ml solution in chloroform/methanol (2:1) under nitrogen in the dark.

7.3 ANALYTICAL PROCEDURES

7.3.1 SDS Gel Electrophoresis

10% SDS slab gels were prepared essentially according to the method of Panyim and Chalkley (1971). The following solutions were made:

Running Gel

Sol A - 60% acrylamide
0,4% NN'-methylenebisacrylamide

Sol B - 0,2M glycine, pH 10
0,5% SDS
2% ($^{V}/V$) TEMED

Sol C - 0,25% Ammonium persulphate
16% glycerol

Tray Buffer: 0,25M glycine, pH 10; 0,5% SDS

Sample Application Buffer: 0,01M Tris, pH 6,7; 2% SDS; 10% ($^{V}/V$) glycerol; 1% ($^{V}/V$) 2 mercaptoethanol; 0,1mg/ml bromophenol blue

The running gel was prepared by mixing A, B, and C in the ratio 1:1:4. This was overlayed with a 3% stacking gel pH 6,7.

Samples were solubilized in sample application buffer to give a 1mg/ml solution and heated for 5 minutes at 100^{o}C in a boiling water bath. The gels were run for approximately 3,5 hours at constant voltage (100V) and cooled continuously by a fan during electrophoresis.

7.3.2 Gel Staining for Protein

7.3.2.1 Coomassie Brilliant Blue

Gels were stained for protein for 1 hour with 0,1% Coomassie Brilliant Blue R250 in 50% ($^{V}/v$) methanol, 10% ($^{V}/v$) acetic acid and destained by diffusion in 25% ethanol, 7% acetic acid. Gels were photographed over a light box using a red filter.

7.3.2.2 Silver Staining

The method of Dubray and Bezard (1982) was followed.

1. The gel was fixed overnight in 25% ethanol, 7% acetic acid and washed in 3 changes of distilled water.
2. The gel was then soaked in 10% unbuffered glutaraldehyde at room temperature for 30 minutes. It was then rinsed in distilled water at room temperature overnight.
3. The water was drained off and the gel stained for ten minutes in a freshly prepared solution of 100 ml of ammoniacal silver solution. This solution is prepared by adding 2 ml of concentrated ammonium hydroxide to 28 mls of 0,1 N sodium hydroxide. To this solution is added 5 mls of 20% silver nitrate while stirring. The solution is made up to 100 mls with water.
4. The gel was washed three times for 10 minutes in 500 mls distilled water.
5. The gel was transferred to a freshly prepared solution containing 0,05% citric acid, 0,02% formaldehyde. Development was terminated when the stain reached the desired intensity by washing in Kodafix rapid fixer.
6. Gels were photographed over a light box using a blue filter.

7.3.3 Gel Staining for Glycoproteins

7.3.3.1 Alcian Blue

The method of Wardi et al. (1972) was followed.

Gels were placed in 12,5% acetic acid for 30 minutes, rinsed with water and soaked in 1% periodic acid (in 3% acetic acid) for 1 hour. Excess periodate was removed by repeated washing with water and gels were placed in 0,5% potassium metabisulphite for 30 minutes. Gels were again washed with water and placed for 4 hours in 0,5% Alcian Blue (in 3% acetic acid) - Gels were destained by diffusion in 7% acetic acid.

7.3.3.2 Silver Staining

The method of Dubray and Bezard (1982) was followed.

The gel was fixed overnight in 25% ethanol, 10% acetic acid and then soaked for 30 minutes in 7,5% acetic acid. The gel was placed in 0,7% aqueous periodic acid for one hour and then washed for one hour in several changes of distilled water. Thereafter, steps 3 - 6 outlined in 7.3.2.2 were followed.

7.3.3.3 Fluorescein Labelled Lectins

Gels were stained for carbohydrate using fluorescein labelled Con A or LCH. The gels were fixed overnight in 25% ethanol, 7% acetic acid and then equilibrated in 0,1M NaCl, 0,01M phosphate pH 7,5 and stained in a 1mg/ml solution of lectin in the same buffer for 3 hours. 0,5mM Ca^{++} and Mn^{++} were included in the staining solution. Non-specific binding was monitored by staining in the presence of 0,1M of α-D-methyl-glucopyranoside or 0,1M glucose.

Gels were destained by diffusion, until the background was free of fluorescence. Gels were scanned for fluorescence on a Vitatron TLD 100 densitometer with Hanau ST 41 Mercury lamp as source or were photographed over a short wave (mainly 254nm) transilluminator (UV Products) through a Wratten type 61 filter.

7.3.4 Determination of S Values

S values were determined on a linear 5 - 20% sucrose gradient by comparison with standards of known molecular weight. Samples and gradient were buffered with 0,01M Tris pH 7,5; 0,15M NaCl; 1mM Na_2 EDTA; 1mM β-mercaptoethanol.

Linear 5 - 20% sucrose gradients were formed in 5ml cellulose nitrate tubes using a Beckman gradient former. Ovalbumin, myoglobin and bovine serum albumin (BSA) (100 μg) were run as standards. In the case of TA-receptor complex, a 10 000 dpm aliquot was applied to the gradient. The sample of TA-receptor complex used for S value determination was free of BSA.

Samples were centrifuged for 19 hours at 60krpm in a Beckman SW65 rotor. Gradients were analysed on an ISCO Gradient Analyser (Model 640)

coupled to an ISCO Absorbance Monitor (UA-5). Where required, 0,2ml samples were collected and assayed for radioactivity.

7.3.5 Determination of Phosphorus

Phosphorus was determined according to the method of Chen et al., (1972).

Reagents:

A. Ascorbic acid 10% (w/v) in H_2O
B. Ammonium molybdate 2,5% (w/v) in H_2O
C. H_2SO_4 6N
D. 1 vol C + 2 vol H_2O + 1 vol A + 1 vol B

All glassware was washed in concentrated H_2SO_4 and concentrated HNO_3. Samples were dried under nitrogen in acid cleaned reflux tubes. 1ml of concentrated H_2SO_4 was added and the tube heated on a heating block until white fumes of sulphur trioxide appeared. 2 drops (0,5ml) of 70% $HClO_4$ were added and the tubes were allowed to reflux for 30 minutes after which time the solution had become colourless.

The refluxed sample was made up to 4 mls with H_2O and 4 mls of reagent D was added. Tubes were sealed, incubated at 37°C for 2 hours and read at 578nm.

A standard curve was drawn up using inorganic phosphate as standard.

7.3.6 DNA Determination

DNA was determined according to the method of Burton (1956) using salmon DNA (Sigma) as standard.

7.3.7 Protein Determination

Protein was determined according to the method of Lowry et al. (1951) using crystalline bovine serum albumin (Sigma) as standard.

7.3.8 Liquid Scintillation Counting

Samples containing tritiated triamcinolone acetonide were assayed for radioactivity in a Beckman LS 250 Liquid Scintillation Counter, using the following scintillator solution: 300g Triton X-100, 50 ml of 20% SDS, 5g 2,5 diphenyl-1, 3-oxazol (PPO) made up to 1 litre with toluene.

7.3.9 Radioiodination of Nuclei, Nuclear Envelope and Nuclear Protein Matrix

A modification of the method of Richardson and Maddy (1980a) was followed.

Nuclear envelopes or nuclear matrix were suspended in 500 µl 0,25M sucrose, 20µM KI, 10mM Tris pH 7,2. To this was added 30 µl of 1mM H_2O_2 and 10 µl of $Na^{125}I$ (100 mCi/ml).

To initiate the reaction, 50 µl of Sepharose bound lactoperoxidase was added. The mixture was incubated with gentle shaking for 2 hours at $+4^{o}C$ or 15 minutes at room temperature and the reaction terminated by addition of 100µl of 20mM Na metabisulphite in 0,25M sucrose, 10mM Tris pH 7,2. In the case of nuclei the same procedure was followed but nuclei were suspended in 0,25M sucrose TKM and KI added to a final concentration of 20 µM. Sepharose was removed by passing the suspension through a 61 µm mesh nylon net. Unreacted ^{125}I was removed by pelleting the nuclei or nuclear envelopes and removing the supernatant. The pellet was washed until the supernatant contained 0,5% of radioactivity present in the pellet.

7.3.10 Radioiodination of Insulin

Insulin was iodinated by the same method employed for nuclear envelope. Unbound iodine was separated from iodinated insulin by chromatography on Sephadex G-25 where unreacted iodine eluted in the inner volume.

7.3.11 Autoradiography

Iodinated samples were solubilized in SDS sample application buffer and

run on 10% polyacrylamide gels as described (7.3.1). The gels were stained with Coomassie Brilliant Blue and dried under vacuum. Autoradioagraphy was performed in X-ray cassettes using Kodak X-omat or Chronex 4 X-ray film. Exposure time varied between 5 hours and 1 month depending on the activity of the sample. The films were developed using Kodak D 19 developer.

7.3.12 Thin Layer Chromatography

Lecithin purity and the lipid composition of the nuclear envelope were assessed by thin layer chromatography on silica gel H plates with a thickness of 0.5 mm. 20 - 50 µg of lecithin or 100 - 500 µg of total envelope lipid were spotted on the plate. The plates were developed using chloroform : methanol : acetic acid : water (25:15:4:2) and the lipids visualized with iodine vapour.

7.3.13 Purification of 2 - Chloroethanol

2 - Chloroethanol obtained from Merck had appreciable absorbance in the range 230 - 250 nm and was purified by passing it over a charcoal column and by distillation. This was sufficient to yield a product with $OD_{230} \leq 0,1$. The apparent pH of a 2 - chloroethanol : water mixture (9:1) was usually in the range 1 - 2.

7.3.14 Determination of Carbohydrate

Carbohydrate was determined using the anthrone reaction. Anthrone reagent was prepared by dissolving anthrone in 78% sulfuric acid to give a 0,2% solution. 9 mls of anthrone reagent were added to 1 ml of sample at $4^{o}C$. Tubes were incubated in a boiling water bath for exactly ten minutes and then cooled at once on ice. Absorbance at 578 nm was measured exactly one hour later. A standard curve was prepared using glycogen as standard.

7.4 INCUBATION AND RECONSTITUTION CONDITIONS

7.4.1 Incubation of Nuclear and Other Membranes with Activated Cytoplasmic TA-Receptor Complex

All incubations were carried out at +4°C in TGA buffer (unless otherwise indicated) in small plastic vials. The amount of membrane used per incubation varied between 120 and 150 µg with respect to protein while the amount of TA-receptor complex varied between 0,5 - 1,0 p mole (3 - 6 nCi). Incubations were carried out in a total volume of 300µl for 1 hour. Vials were rotated throughout the incubation on a Coulter mixer. The incubates were then layered over a 25 - 50% (w/v) sucrose TGA gradient and centrifuged for 3,5 hours at 170 000 g in a SW40Ti Rotor. The gradients were analysed on an ISCO Density Gradient Fractionator using a 280 nm filter. 0,4 ml fractions were collected and assayed for radioactivity. Where incubations took place in the presence of 0,3 M NaCl, the gradient was also made 0,3 M in NaCl.

7.4.2 Incubation of Nuclei and Chromatin with Activated Cytoplasmic TA-Receptor Complex

Incubation conditions for nuclei and chromatin were identical to those for membranes. The amount of nuclei or chromatin used per incubation was that amount yielding approximately 100 µg nuclear envelope. Prior to incubation with nuclei or chromatin aggregated material was removed from TA-receptor complex aliquots by centrifugation at 100 000 g for 1 hour and only the soluble component was used in the incubation. After incubation the nuclei or chromatin were pelleted by centrifugation, washed with TGA buffer and both pellet and supernatant were assayed for radioactivity. Nonspecific pelleting was monitored by incubation of TA receptor in the absence of nuclei or chromatin.

7.4.3 Scatchard Analysis

Scatchard analysis was performed on data obtained from saturation curves. In all cases the concentration of TA-receptor complex in each incubation was increased while the amount of acceptor (membrane/chromatin/nuclei) was kept constant.

After 1 hour at +4°C incubates were centrifuged at 10 000 g for 30 minutes and the supernatants and pellets counted. Nonspecific pelleting was monitored by incubating without acceptor.

7.4.4 Incubations with DNase

DNase 1 (Merck, 2 000 E/mg) was incubated with membrane samples for various lengths of time at 0°C and 25°C in 0,01 M Na Phosphate pH 7,5. The membrane was then pelleted by centrifugation and the supernatant assayed for DNA.

7.4.5 Reconstitution of Lipid Free Envelope Proteins with Lecithin or Native Envelope Lipid

1 - 5 mg of purified lecithin or lipid extracted from nuclear envelopes was dried from chloroform:methanol by a stream of nitrogen. An aliquot of protein (100 - 300 µl) in 2 - chloroethanol was added to the lipid. This resulted in immediate solubilization of the lipid. The solution was then immediately dialysed against 0,1 M NaCl, 0,01 M phosphate pH 7,5 overnight. The contents of the dialysis bag were removed and analysed by sucrose gradient centrifugation.

7.4.6 Reconstitution of Detergent Extracted Nuclear Envelopes with Phospholipid

Lecithin or extracted envelope lipid were dried under nitrogen in a glass tube. Detergent extracts of nuclear envelope were made 70% in chloroethanol and added directly to the lipid at 4°C. Detergent insoluble fractions were taken up in chloroethanol:0,01 M phosphate pH 7,5 (9:1) and added to the lipid. The samples were immediately dialysed against 0,1 M NaCl, 0,01 M phosphate pH 7,5 overnight. Samples were analysed by sucrose gradient centrifugation.

REFERENCES

Aaronson, R.P. and Blobel, G., (1974) On the attachment of the nuclear pore complex. J. Cell Biol. 62, 746 - 754.

Aaronson, R.P. and Blobel, G., (1975) Isolation of nuclear pore complexes in association with a lamina. Proc. Nat. Acad. Sci. U.S.A. 72, 1007 - 1011.

Adelman, M.R., Blobel, G. and Sabatini, D.D., (1974) in Methods in Enzymology Vol. 31, 210 - 214. (Eds. S. Fleischer and S. Packer), Academic Press, N.Y.

Adolph, K.W., Cheng, S.M., Paulson, J.R. and Laemmli, U.K., (1977) Isolation of a protein scaffold from mitotic HeLa cell chromosomes. Proc. Nat. Acad. Sci. U.S.A., 74, 4937 - 4951.

Adolph, K.W., (1980) Organization of chromosomes in HeLa cells: isolation of histone depleted nuclei and nuclear scaffolds. J. Cell Science 42, 291 - 304.

Agarwal, B.B.L. and Goldstein, I.J., (1967) Protein-carbohydrate interaction. VI. Isolation of Concanvalin A by specific adsorption on cross-linked dextran gels. Biochim. Biophys Acta 147, 262 - 271.

Agarwal, M.K., (1976) Evidence that natural versus synthetic steroid hormones bind to physiochemically distinct cellular receptors. Biochim. Biophys. Res. Comm. 73, 767 - 772.

Agarwal, M.K., (1977) Further characterization and subunit nature of mineralo- and gluco-steroid receptors from rat liver and kidney. Int. J. Biochem. 8, 877 - 881.

Agutter, P.S., (1972) The isolation of the envelopes of rat liver nuclei. Biochim. Biophys. Acta. 255, 397 - 401.

Agutter, P.S. and Birchall, K., (1979) Functional differences between mammalian nuclear protein matrices and pore-lamina complex laminae. Exp. Cell, Res. 124, 453 - 460.

Agutter, P.S. and Richardson, J.C.W., (1980) Nuclear non-chromatin proteinaceous structures: their role in the organization and function of the interphase nucleus. J. Cell Science 44, 395 - 435.

Allfrey, V.G., Johnson, E.M., Kam, J. and Vidali, G., (1973) Phosphorylation of nuclear proteins at times of gene activation. Protein phosphorylation in Control Mechanisms. (Eds. Huising, F. and Lee, E.Y.C.), Academic Press, N.Y.

Altman, P.L. and Dittmer, D.S., Eds., (1964) Biological Data Book p. 399. Fed. of American Societies for Experimental Biology, Bethesda, Md.

André, J. and Rochefort, H., (1975) In vitro binding of estrogen receptor to DNA: absence of saturation at equilibrium. Febs. Lett. 50, 319 - 323.

Andreasen, P.A., (1981) A specific adenine nucleotide effect on the rat liver glucocorticoid receptor demonstrated by aqueous two-phase partitioning. Biochim. Biophys. Acta. 676, 205 - 212.

Aronson, N.N. and Touster, O., (1974) in Methods in Enzymology, Vol. 31, pp. 94 - 101. (Eds. Fleischer, S. and Packer, S.), Academic Press, N.Y.

Ashley, T., (1974) Attachment of mitotic prophase chromosome ends to the nuclear membrane. J. Cell. Biol. 63, 11a.

Atger, M. and Milgrom, E., (1976a) Mechanism and kinetics of the thermal activation of glucocorticoid hormone-receptor complex. J. Biol. Chem. 251, 4758 - 4762.

Atger, M. and Milgrom, E., (1976b) Chromatographic separation on phosphocellulose of activated and nonactivated forms of steroid-receptor complex. Purification of the activated complex. Biochemistry 15, 4298 - 4304.

Atger, M. and Milgrom, E., (1978) Interaction of glucocorticoid-receptor complexes with rat liver nuclei. Biochim. Biophys. Acta. 539, 41 - 53.

Bailly, A., Savouret, J., Sallas, N. and Milgrom, E., (1978) Factors modifying the equilibrium between activated and non-activated forms of steroid-receptor complex. Eur. J. Biochem. 88, 623 - 632.

Bailly, A., Le Fevre, B., Savouret, J. and Milgrom, E., (1980) Activation and changes in sedimentation properties of steroid receptors. J. Biol. Chem. 255, 2729 - 2734.

Balhorn, R., Rieke, O. and Chalkley, R., (1971) Rapid electrophoretic analysis for histone phosphorylation. A reinvestigation of phosphorylation of lysine-rich histone during rat liver regeneration. Biochemistry 10, 3952 - 3958.

Ballard, P.L., Baxter, J.D., Higgins, S.J., Rousseau, G.G. and Tomkins, G.M., (1974) General presence of glucocorticoid receptors in mammalian tissue. Endocrinology 94, 998 - 1002.

Barnett, C.A., Schmidt, T.J. and Litwack, G., (1980) Effects of calf intestinal alkaline phosphatase, phosphatase inhibitors, and phosphorylated compounds on the rate of activation of glucocorticoid-receptor complexes. Biochemistry 19, 5446 - 5455.

Barrack, E.R., Hawkins, E.F., Allen, S.L., Hicks, L.L. and Coffey, D.S., (1977) Concepts related to salt resistant estradiol receptors in rat uterine nuclei: nuclear matrix. Biochem. Biophys. Res. Comm. 79, 829 - 836.

Barrack, E.R. and Coffey, D.S., (1980) Specific binding of estrogens and androgens to the nuclear matrix of sex hormone responsive tissues. J. Biol. Chem. 15, 7265 - 7275.

Baxter, J.D. and Tomkins, G.M., (1970) The relationship between glucocorticoid binding and tyrosine aminotransferase induction in hepatoma tissue culture cells. Proc. Natl. Acad. Sci. U.S.A., 65, 709 - 715.

Baxter, J.D., Rousseau, G.G., Bensen, M.C., Garcea, R.L., Ito, J. and Tomkins, G.M., (1972) Role of DNA and specific cytoplasmic receptors in glucocorticoid action. Proc. Nat. Acad. Sci. U.S.A. 65, 709 - 715.

Beato, M., Brandle, W., Biesewig, D. and Sekeris, C.E., (1970) On the mechanism of hormone action. XVI. Transfer of (1,2 - $^{3}H_{2}$) cortisol from the cytoplasm to the nucleus of rat liver cells. Biochim. Biophys. Acta 208, 125 - 136.

Beato, M. and Feigelson, P., (1972) Glucocorticoid-binding proteins of rat liver cytosol. I. Separation and identification of the binding proteins. J. Biol.Chem. 247, 7890 - 7896.

Beato, M., Kalimi, M., Beato, W. and Feigelson, P., (1974) Interaction of glucocorticoids with rat liver nuclei: effect of adrenalectomy and cortisol administration. Endocrinology 94, 377 - 387.

Berezney, R. and Crane, F.L., (1972) Characterization of electron transport activity in bovine liver nuclear membranes. J. Biol. Chem. 247, 5562 - 5568.

Berezney, R. and Coffey, D.S., (1974) Identification of a nuclear protein matrix. Biochem. Biophys. Res. Comm. 60, 1410 - 1417.

Berezney, R. and Coffey, D.S., (1976) The nuclear protein matrix: isolation, structure and functions. Adv. Eng. Reg. 14, 63 - 100.

Berezney, R. and Coffey, D.S., (1977) Nuclear matrix: isolation and characterization of a framework structure from rat liver nuclei. J. Cell. Biol. 73, 616 - 637.

Berezney, R., Basler, J., Hughes, B.B. and Kaplan, S.C. (1979) Isolation and characterization of the nuclear matrix from Zajdela ascites hepatoma cells. Cancer Research 39, 3031 - 3039.

Berezney, R. and Buchholtz, L.A., (1981a) Dynamic association of replicating DNA fragments with the nuclear matrix of regenerating liver. Exp. Cell Res. 132, 1 - 13.

Berezney, R. and Buchholtz, L.A., (1981b) Isolation and characterization of rat liver nuclear matrices containing high molecular weight deoxyribonucleic acid. Biochemistry 20, 4995 - 5002.

Blobel, G. and Potter, van R., (1966) Nuclei from rat liver: isolation method that combines purity with high yield. Science 154, 1662 - 1665.

Bloom, E., Matulich, D.T., Lan, N.C., Higgins, S.J., Simons, S.S. and Baxter, J.D., (1980) Nuclear binding of glucocorticoid receptors: relationship between cytosol binding, activation and the biological response. J. Steroid. Biochem. 12, 175 - 184.

Bonner, J., Chalkley, G.R., Dahmus, M., Fambrough, D., Fujimura, F., Huang, R.C., Huberman, J., Jensen, R., Marushige, K., Ohlenbusch, H., Olivera, B. and Widholm, J., (1968) in Methods in Enzymology, Vol 12, pp. 3 - 5. (Eds. Grossman, L. and Moldave, K.), Academic Press, N.Y.

Bonner, J., Sala-Teepat, J.M., Pearson, W.R. and Wu, J., (1978) in The Cell Nucleus, Vol 6, pp. 369 - 407. (Ed. Busch, H.), Academic Press, N.Y.

Bornens, M. and Kasper, C.B., (1973) Fractionation and partial characterization of proteins of the bileaflet nuclear membrane from rat liver. J. Biol. Chem. 248, 571 - 579.

Bornens, M., (1977) Solubilization of chromatin with heparin and the isolation of nuclear membranes. Methods Cell Biol. 15, 163 - 175.

Bornens, M. and Courvalin, J., (1978) Isolation of nuclear envelopes with polyanions. J. Cell Biol. 76, 191 - 206.

Bugany, H. and Beato, M., (1977) Binding of partially purified glucocorticoid receptor of rat liver to chromatin and DNA. Mol. Cell. Endocrin. 7, 49 - 66.

Bulanyi, G.S. and Oliver, I.T., (1976) Cytosol protein aggregation and dexamethasone binding to isolated rat liver nuclei. Febs. Lett. 70, 33 - 36.

Buller, R.E., Schwartz, R.J., Schrader, W.T. and O'Malley, B.W., (1976) Progesterone-binding components of the chick oviduct XII. In vitro effect of receptor subunits on gene transcription. J. Biol. Chem 251, 5178 - 5186

Burton, K., (1956) A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J. 62, 315 - 323

C.R.C. Handbook of Biochemistry (1968) 2nd edition. (Ed. Sober, H.A.) Chemical Rubber Company, Cleveland, Ohio.

Cabradilla, C.D. and Toliver, A.P., (1975) S-phase dependent forms of DNA-nuclear membrane complexes in HeLa cells. Biochim. Biophys. Acta 402, 188 - 198.

Carlstedt-Duke, J., Gustaffson, J. and Wrange, O., (1977) Formation and characteristics of hepatic dexamethasone receptor complexes of different molecular weight. Biochim. Biophys. Acta 497, 507 - 524.

Chen, P.S., Toribara, T.Y. and Warner, H., (1956) Microdetermination of phosphorus. Anal. Biochem 28, 1756 - 1758.

Chong, M.T. and Lippman, M.E., (1982) Effects of RNA and ribonuclease on the binding of estrogen and glucocorticoid receptors from MCF-7 cells to DNA-cellulose. J. Biol. Chem. 257, 2996 - 3002.

Cidlowski, J.A. and Munck, A., (1978) Comparison of glucocorticoid-receptor complex binding to nuclei and DNA cellulose: evidence for different forms of interaction. Biochim. Biophys. Acta 543, 545 - 555.

Clawson, G.A., James, J., Woo, C.H., Friend. D.S., Moody, D. and Smuckler, E.A., (1980) Pertinence of nuclear envelope nucleoside triphosphatase activity to ribonucleic acid transport. Biochemistry 19, 2748 - 2756

Clay, W.F., Katterman, F.R.H. and Bartels, P.G., (1975) Chromatin and DNA synthesis associated with nuclear membrane in germinating cotton. Proc. Nat. Acad. Sci. U.S.A. 72, 3134 - 3138.

Climent, F., Bugany, H. and Beato, M., (1976) Partial purification of the activated glucocorticoid receptor of rat liver. Febs. Lett. 66, 317 - 321.

Climent, F., Doenecke, D. and Beato, M., (1977) Properties of the partially purified activated glucocorticoid receptor of rat liver. Binding to chromatin subunits. Biochemistry 16, 4694 - 4703.

Comings, D.E. and Kakefuda, T., (1968) Initiation of deoxyribonucleic acid replication at the nuclear membrane in human cells. J. Mol. Biol. 33, 225 - 229.

Comings, D.E. and Okada, T.A., (1970a) Association of chromatin fibres with the annuli of the nuclear membrane. Exp. Cell Res. 62, 293 - 302.

Comings, D.E. and Okada, T.A., (1970b) Condensation of chromosomes onto the nuclear membrane during prophase. Exp. Cell. Res. 63, 471 - 473.

Comings, D.E. and Okada, T.A., (1970c) Association of nuclear membrane fragments with metaphase and anaphase chromosomes as observed by whole mount electron microscopy. Exp. Cell Res. 63, 62 - 68.

Comings, D.E. and Okada, T.A., (1976) Nuclear proteins 3. Fibrillar nature of nuclear matrix. Exp. Cell Res. 103, 341 - 360.

Compton, J.G., Schrader, W.T. and O'Malley, B.W., (1983) DNA sequence preference of the progesterone receptor. Proc. Nat. Acad. Sci. U.S.A. 80, 16 - 20

Dabeva, M.D., Petrov, P.T., Stoykova, A.S. and Hadjiolov, A.A., (1977) Contamination of detergent-purified rat liver nuclei by cytoplasmic ribosomes. Exp. Cell Res. 108, 467 - 471.

Dahmer, M.K., Quasney, M.W., Bissen, S.T. and Pratt, W.B., (1981) Molybdate permits resolution of untransformed glucocorticoid receptors from the transformed state. J. Biol. Chem. 256, 9401 - 9405.

Defer, N., Dastague, B. and Kruh, J., (1974a) Direct binding of corticosterone and estradiol to rat liver nuclear nonhistone proteins. Biochimie 56, 559 - 566.

Defer, N., Dastague, B. and Kruh, J., (1974b) Rat liver chromatin nonhistone proteins and glucocorticoid binding. Biochimie 56, 1549 - 1557.

Detke, S. and Keller, J.M., (1982) Comparison of the proteins present in HeLa cell interphase nucleoskeletons and metaphase chromosome scaffolds. J. Biol. Chem. 257, 3905 - 3911.

Disorbo, D.M., Phelps, D.A. and Litwack, G., (1980) Chemical probes of amino acid residues affect the active sites of the glucocorticoid receptor. Endocrinology 106, 922 - 929.

Dolan, K.P., Diaz-gil, J.J. and Litwack, G., (1980) Interaction of pyridoxal 5'-phosphate with the liver glucocorticoid receptor-DNA complex. Arch. Biochem. Biophys., 201, 476 - 485.

Dubray, G. and Bezard, G., (1982) Highly sensitive periodic acid silver stain for 1,2 diol groups of glycoproteins and polysaccharides in polyacrylamide gels. Anal. Biochem. 119, 325 - 329.

Du Praw, E.J., (1968)
In Cell and Molecular Biology, p. 474, Academic Press, N.Y.

Dwyer, N. and Blobel, G., (1976) A modified procedure for the isolation of a pore complex-lamina fraction from rat liver nuclei. J. Cell Biol. 70, 581 - 591.

Eckhardt, A.E., Hayes, C.E. and Goldstein, I.J., (1976) A sensitive fluorescent method for the detection of glycoproteins in polyacrylamide gels. Anal. Biochem. 73, 192 - 197.

Eisen, H.J. and Glinsmann,W., (1976) Partial purification of the glucocorticoid receptor from rat liver: a rapid two step procedure using DNA-cellulose. Biochem. Biophys. Res. Comm 70, 367 - 372.

Eisen, H.J. and Glinsmann, W.H., (1978) Maximizing the purification of the activated glucocorticoid receptor by DNA-cellulose chromatography. Biochem. J. 171, 177 - 183.

Eisen, H.J., (1980) An antiserum to the rat liver glucocorticoid receptor. Proc. Nat. Acad. Sci. U.S.A. 77, 3893 - 3897.

Eisen, H.J., Schleenbaker, R.E. and Simons, S.S., (1981) Affinity labelling of the rat liver glucocorticoid with dexamethasone 21-mesylate. J. Biol. Chem. 256, 12920 - 12925.

Ely, S., D'Arcy, A. and Jost, E., (1978) Interaction of antibodies against nuclear envelope-associated proteins from rat liver nuclei with rodent and human cells. Exp. Cell Res. 116, 325 - 331.

Eytan, G. and Kanner, B.I., (1978) in Receptors and Recognition, Vol. 6, pp. 65 - 105, (Eds. Cuatrecasas,P. and Greaves, M.), Chapman and Hall.

Feldman, D., Funder, J. and Loose, D., (1978) Is the glucocorticoid receptor identical in various target organs? J. Steroid Biochem. 9, 141 - 145.

Fisher, P.A., Berrios, M. and Blobel, G., (1982) Isolation and characterization of a proteinaceous subnuclear fraction composed of nuclear matrix, peripheral lamina and nuclear pore complexes from embryos of Drosophila melanogaster. J. Cell Biol. 92, 674 - 686.

Franke, W.W., Deumling, B., Ermen, B., Jarasch, E.D. and Kleinig, H., (1970) Nuclear membranes from mammalian liver 1. Isolation procedure and general characterization. J. Cell. Biol. 46, 377 - 395.

Franke, W.W. and Scheer, U., (1970) The ultrastructure of the nuclear envelope of amphibian oocytes : a reinvestigation. J. Ultrastructure Res. 30, 317 - 327

Franke, W.W., Deumling, B., Zentgraf, H., Falk, H. and Rae, P.M.M., (1973) Nuclear membranes from mammalian liver. IV. Characterization of membrane-attached DNA. Exp. Cell Res. 81, 365 - 392.

Franke, W.W., (1974) Structure,biochemistry and functions of the nuclear envelope. Int. Rev. Cytology, Supp. 4, 71 - 236.

Franke, W.W. and Scheer, U., (1974) Structures and functions of the nuclear envelope. In The Cell Nucleus, Vol. 1, pp. 279 - 347, (Ed. Busch, H.), Academic Press, N.Y.

Franke, W.W., Keenan, T.W., Stadler, J., Genz, R., Jarasch, E. and Kartenbeck, J., (1976) Nuclear membranes from mammalian liver. VII. Characteristics of highly purified membranes in comparison with other membranes. Cytobiologie 13, 28 - 56.

Franke, W.W., Kleinschmidt, J.A., Spring, H., Krohne, G., Grund, C., Trendelenburg, M.F., Stoehr, M. and Scheer, U., (1981) A nucleolar skeleton of protein filaments demonstrated in amplified nucleoli of Xenopus laevis. J. Cell Biol. 90, 289 - 299.

Frederiks, W.M., James, J., Arnouts, C., Broekhoven, S. and Morreau, J., (1978) The influence of Triton X-100 on the nuclear envelope of the isolated liver cell nuclei. Cytobiologie 18, 254 - 271.

Frederik, P.M., van der Molen, H.J., Klepper, D. and Galjaard, H., (1977) Electron-microscopic autoradiography of tritiated testosterone in rat testis. J. Cell Science 26, 339 - 346.

Galchevagargova, Z., Petrov, P. and Dessev, G., (1982) Effect of chromatin decondensation on the intranuclear matrix. Eur. J. Cell. Biol. 28, 155 - 159.

Galli, M.G., Chiatante,D., Sparvoli, E. and Mariani, D., (1978) Localization of late replicating DNA and nuclear membrane. Chromosoma 67, 285 - 291.

Gerace, L., Blum, A. and Blobel, G., (1978) Immunocytochemical localization of the major polypeptides of the nuclear pore complex lamina fraction. J. Cell Biol. 79, 546 - 566.

Gerace, L. and Blobel, G., (1980) The nuclear envelope lamina is reversibly depolymerized during mitosis. Cell 19, 277 - 287.

Giannopolous, G., Mulay, S. and Solomon, S., (1973) Glucocorticoid receptors in the lung. J. Biol. Chem. 248, 5016 - 1023.

Giannopolous, G., (1975) Glucocorticoid receptors in the lung: mechanism of specific glucocorticoid uptake by fetal rabbit lung nuclei. J. Biol. Chem. 250, 2896 - 2903.

Giannopolous, G., (1977) Binding of glucocorticoids to liver nuclei and chromatin of fetal, immature and adult rats. Steroids 29, 309 - 329.

Giese, G. and Wunderlich, F., (1980) Increased fluidity and loss of temperature and Ca^{++}/Mg^{++} sensitivity in nuclear membranes upon removal from the membrane-associated nuclear matrix proteins. J. Biol. Chem. 255, 1716 - 1721.

Gilmour, R.S. and Paul, J., (1975) Chromosomal proteins, their regulation and gene expression. Proc. Fla. College Mol. Biol. 1975, 19 - 33.

Gilmour, R.S., Windass, J.D., Affara, N. and Paul, J., (1975) Control of transcription of the globin gene. J. Cell Phys. 85, 449 - 458.

Goidl, J.A., Cake, M.H., Dolan, K.P., Parchman, L.G. and Litwack, G., (1977) Activation of the rat liver glucocorticoid-receptor complex. Biochemistry 16, 2125 - 2130.

Goldberg, A.H., Yeoman, L.C. and Busch, H., (1978) Chromatin-associated glycoproteins of normal rat liver and Novikoff Hepatoma Ascites cells. Cancer Research 38, 1052 - 1056.

Govindan, M.V. and Sekeris, C.E., (1976) Partial purification of rat liver glucocorticoid binding proteins by affinity chromatography. Steroids 28, 499 - 507.

Govindan, M.V. and Sekeris, C.E., (1978) Purification of two dexamethasone binding proteins from rat-liver cytosol. Eur. J. Biochem. 89, 95 - 104.

Govindan, M.V., (1980) Isolation and characterization of rat liver nuclear glucocorticoid receptor. Biochim. Biophys. Acta 631, 327 - 333.

Govindan, M.V. and Manz, B., (1980) Three-step purification of glucocorticoid receptors from rat liver. Eur. J. Biochem. 108, 47 - 53.

Govindan, M.V., Spiess, E. and Majors, J., (1982) Purified glucocorticoid receptor-hormone complex from rat liver cytosol binds specifically to cloned mouse mammary tumour virus long terminal repeats in vitro. Proc. Nat. Acad. Sci. U.S.A. 79, 5157 - 5161.

Gronemeyer, H. and Pongs, O., (1980) Localization of ecdysterone on polytene chromosomes of Drosophila melanogaster. Proc. Nat. Acad. Sci. U.S.A. 77, 2108 - 2112.

Hamana, K. and Iwai, K., (1978) Glucocorticoid-receptor complex binds to nonhistone protein and DNA in rat liver chromatin. J. Biochem. 83, 279 - 286.

Handbook of Biochemistry and Molecular Biology, (1976), Vol. 2, p.273. (Ed. Fasman, Gerald D.), 3rd edition, CRC Press.

Harlow, R., Tolstoshev, P. and Wells, J.R.E., (1972) Membrane - a major source of chromatin-associated RNA and non-histone proteins in avian erythroid cells. Cell. Diff. 2, 341 - 349.

Harris, J.R., (1977) Fractionation of the nuclear envelope. in Membranous Elements and Movements of Molecules: Techniques, Vol. 6, pp. 245 - 250. (Ed. Reid, E.), Horwood, Chichester.

Harris, J.R., (1978) The biochemistry and ultrastructure of the nuclear envelope. Biochim. Biophys. Acta 515, 55 - 104.

Higgins, S.J., Rousseau, G.G., Baxter, J.D. and Tomkins, G.M., (1973a) Early events in glucocorticoid action: activation of the steroid receptor and its subsequent specific nuclear binding studied in a cell-free system. J. Biol. Chem. 248, 5866 - 5872.

Higgins, S. J., Rousseau, G.G., Baxter, J.D. and Tomkins, G.M., (1973b) Nature of nuclear acceptor sites for glucocorticoid- and estrogen-receptor complexes. J. Biol. Chem. 248, 5873 - 5879.

Higgins, S.J., Baxter, J.D. and Rousseau, G.G., (1979) in Glucocorticoid Hormone Action, Chap. 8, pp. 135 - 160. (Eds. Baxter, J.D. and Rousseau, G.G.), Springer Verlag.

Hobart, P., Duncan, R. and Infante, A.A., (1977) Association of DNA synthesis with the nuclear membrane in sea urchin embryos. Nature 267, 542 - 544.

Horvat, A., (1978) Insulin binding sites on rat liver nuclear membranes. J. Cell Phys. 97, 37 - 48.

Howard, I.K. and Sage, H.J., (1969) Isolation and characterization of a phytohemagglutinin from the lentil. Biochemistry 8, 2436 - 2441.

Huang, A.L., Ostrowski, M.C., Beraud, D. and Hagar, G.L., (1981) Glucocorticoid regulation of Ha-MuSV-p21 gene conferred by sequences from mouse mammary tumor virus. Cell 27, 245 - 255.

Huberman, J.A., Tsai, A. and Deich, R.A., (1973) DNA replication sites within nuclei of mammalian cells. Nature 241, 32 - 36.

Hughes, M.R., Compton, J.G., Schrader, W.T. and O'Malley, B.W., (1981) Interaction of the chick oviduct progesterone receptor with deoxyribonucleic acid. Biochemistry 20, 2481 - 2491.

Ichii, S. and Murakami, N., (1978) Nuclei and DNA-binding form of the steroid receptor complex in the liver and the ventral prostrate of rats. Endocrinol. Japan. 25, 539 - 543.

Infante, A.A., Nauta, R., Gilbert, S., Hobart, P. and Firsheim, W., (1973) DNA synthesis in developing sea urchins: role of a DNA-nuclear membrane complex. Nature New Biol. 242, 5 - 8.

Ishii, D.N., Pratt, W.B. and Aronow, L., (1975) Steady-state level of the specific glucocorticoid binding component in mouse fibroblasts. Biochemistry 14, 180 - 186.

Jackson, R.C., (1976) On the identity of nuclear membrane and non-histone nuclear proteins. Biochemistry 15, 5652 - 5656.

Jackson, V. and Chalkley, R., (1974a) The binding of estradiol-17 β to the bovine endometrial nuclear membrane. J. Biol. Chem. 249, 1615 - 1626.

Jackson, V. and Chalkley, R., (1974b) The cytoplasmic estradiol receptors of bovine uterus. J. Biol. Chem. 249, 1627 - 1636.

Jensen, E.V., De Sombre, E.R., Jungbult, P.W., Stumpf, W.E. and Roth, J.L., (1969) In Autoradiography of diffusable substances, pp. 81 - 97, (Eds. Roth, L.J. and Stumpf, W.E.), Academic Press, N.Y.

John, J.K. and Moudgil, V.K., (1979) Activation of glucocorticoid receptor by ATP. Biochem. Biophys. Res. Comm. 90, 1242 - 1248.

Johnson, L.K., Baxter, J.D. and Rousseau, G.G., (1979) in Glucocorticoid Hormone Action, Chap. 17, pp. 305 - 326, (Eds. Baxter, J.D. and Rousseau, G.G.), Springer Verlag.

Jones, T.R. and Bell, P.A., (1982) Glucocorticoid-receptor interactions. Discrimination between glucocorticoid agonist and antagonists by means of receptor-binding kinetics. Biochem. J. 204, 721 - 729.

Kalimi, M., Colman, P. and Feigelsen, P., (1975) The "activated" hepatic glucocorticoid-receptor complex: its generation and properties. J. Biol. Chem. 250, 1080 - 1086.

Kallos, J., (1977) Photochemical attachment of cyclic AMP binding proteins to the nuclear genome. Nature 265, 705 - 710.

Kashnig, D.M. and Kasper, C.B., (1969) Isolation, morphology and composition of the nuclear membrane from rat liver. J. Biol. Chem. 244, 3786 - 3792.

Kasper, C.B., (1974) Chemical and biochemical properties of the nuclear envelope. In The Cell Nucleus, Vol. 1, pp. 349 - 384, (Ed. Busch, H.) Academic Press, N.Y.

Kaufmann, S.H., Coffey, D.S. and Shaper, J.H., (1981) Considerations in the isolation of rat liver nuclear matrix, nuclear envelope and pore complex lamina. Exp. Cell Res. 132, 105 - 123.

Kawasaki, T. and Yamashina, I., (1972) Isolation and characterization of glycopeptides from rat liver nuclear membrane. J. Biochem. 72, 1517 - 1525.

Kay, R.R., Fraser, D. and Johnston, I.R., (1972) A method for the rapid isolation of nuclear membranes from rat liver. Characterization of the membrane preparation and its associated DNA polymerase. Eur. J.. Biochem. 30, 145 - 154.

King, R.J.B., Gordon, J. and Steggles, A.W., (1969) The properties of a nuclear acidic protein fraction that binds 6,7 - ^{3}H oestradiol 17 - β Biochem. J. 114, 649 - 657.

Koblinsky, M., Beato, M., Kalimi, M. and Feigelsen, P., (1972) Glucocorticoid binding proteins of rat liver cytosol. II. Physical characterization and properties of the binding proteins. J. Biol. Chem 247, 7807 - 7904.

Kornberg, T., Lockwood, A. and Worcel, A., (1974) Replication of the Escherichia coli chromosome with a soluble enzyme system. Proc. Nat. Acad. Sci. U.S.A. 71, 3189 - 3193.

Kramer, R., Schlatter, Ch. and Zahler, P., (1972) Preferential binding of sphingomyelin by membrane proteins of the sheep red cell. Biochim. Biophys. Acta 282, 146 - 156.

Krieger, N.S., Middlebrook, J.L. and Aronow, L., (1976) Effect of salt on the reversibility of glucocorticoid receptor binding. J. Steroid Biochem. 7, 395 - 399.

Krohne, G., Franke, W.W., Ely, S., D'Arcy, A. and Jost, E., (1978) Localization of a nuclear envelope-associated protein by indirect immunofluorescence microscopy using antibodies against a major polypeptide from rat liver fractions enriched in nuclear envelope-associated material. Cytobiologie 18, 22 - 38.

Krohne, G., Franke, W.W. and Scheer, U., (1978) The major polypeptides of the nuclear pore complex. Exp. Cell Res. 116, 85 - 102.

Krohne, G., Dabauvalle, M. and Franke, W.W., (1981) Cell type-specific differences in protein composition of nuclear pore complex-lamina structures in oocytes and erythrocytes of Xenopus laevis. J. Mol. Biol. 151, 121 - 141.

Kuzmina, S., Buldyaeva, T., Troitskaya, L. and Zbarsky, I., (1981) Characterization and fractionation of rat liver nuclear matrix. Eur. J. Cell. Biol. 25, 225 - 232.

Lam, K.S. and Kasper, C.B., (1979a) Electrophoretic analysis of three major nuclear envelope polypeptides. J. Biol. Chem. 254, 11713 - 11720.

Lam, K.S. and Kasper, C.B., (1979b) Selective phosphorylation of a nuclear envelope polypeptide by an endogenous protein kinase. Biochemistry 18, 307 - 311.

Lampert, F., (1971) Attachment of human chromatin fibres to the nuclear membrane, as seen by electron microscopy. Humangenetik 13, 285 - 295.

Leach, K.L., Grippo, J.F., Housley, P.R., Dahmer, M.K., Salive, M.E. and Pratt, W.B., (1982) Characteristics of an endogenous glucocorticoid-receptor stabilizing factor. J. Biol. Chem. 257, 381 - 388.

Lefebvre,Y.A. and Novsad, Z., (1980) Binding of androgens to a nuclear-envelope fraction from the rat ventral prostrate. Biochem. J. 186, 641 - 647.

Lefebvre, Y.A. and Morante, S.J., (1982) Binding of dihydrotestosterone to a nuclear envelope fraction from the male rat liver. Biochem. J. 202, 225 - 230.

LeFevre, B., Bailly, A., Sallas, N. and Milgrom, E., (1979) Activated steroid receptor complex: comparison of assays using DNA-cellulose or homologous nuclei. Biochim. Biophys. Acta. 585, 266 - 272.

Leibowitz, P.J. and Schaechter, M., (1975) Attachment of bacterial chromosome to cell membrane. Int. Rev. Cytol. 41, 1 - 28.

Lenard, J. and Singer, S.J., (1966) Protein conformation in cell membrane preparations as studied by optical rotatory dispersion and circular dichroism. Proc. Natl. Acad. Sci. U.S.A. 56, 1828 - 1835.

Liao, S., Liang, T. and Tymoczko, J.L., (1973) Ribonucleoprotein binding of steroid-receptor complexes. Nature New Biol. 241, 211 - 213.

Libby, P., (1973) Histone acetylation and hormone action. Early effects of aldosterone on histone acetylation in rat kidney. Biochem. J. 134, 907 - 912.

Litwack, G., Filler, R., Rosenfield, S.A., Lichtash, N., Wishman, C.A. and Singer, S., (1973) Liver cytosol corticosteroid binder II, a hormone receptor. J. Biol. Chem. 218, 7481 - 7486.

Litwack, G. and Rosenfield, S.A., (1975) Liver cytosol corticosteroid binder 1B, a new binding protein. J. Biol. Chem. 250, 6799 - 6805.

Litwack, G., Cake, M.H., Filler, R. and Taylor, K., (1978) Physical measurements of the liver glucocorticoid receptor. Biochem. J. 169, 445 - 448.

Liu, A.Y. and Greengard, P., (1976) Regulation by steroid hormones of phosphorylation of specific proteins common to several target organs. Proc. Nat. Acad. Sci. U.S.A. 73, 568 - 572.

Long, B.H., Huang, C. and Pogo, A.O., (1979) Isolation and characterization of the nuclear matrix in Friend erythroleukemia cells: chromatin and hnRNA interactions with the nuclear matrix. Cell 18, 1079 - 1090.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R., (1951) Protein measurement with Folin phenol reagent. J. Biol. Chem 193, 265 - 275.

Mainwaring, W.I.P., Symes, E.K. and Higgins, S.J., (1976) Nuclear components responsible for the retention of steroid-receptor complexes, especially from the standpoint of specificity of hormonal responses. Biochem. J. 156, 129 - 141.

Maki, M., Hirose, M and Chiba, H., (1980) Alterations in glucocorticoid receptor conformation by molybdate. J. Biochem. 87, 1851 - 1854.

Malucci, L., (1976) in Concanvalin A as a tool, p.74, (Eds. Bittiger, H. and Schnebli, H.P.), Wiley, London.

Manzoli, F.A., Capitani, S., Maraldi, N.M., Cocco, L. and Barnabei, O., (1978) Chromatin lipids and their possible role in gene expression. A study in normal and neoplastic cells. Adv. in Enz. Reg. 17, 175 - 194.

Marchesi , V.T. and Andrews, E.P., (1971) Glycoprotein isolation from cell membranes with LIS. Science 174, 1247 - 1248.

Markovic, R.D. and Litwack, G., (1980) Activation of liver and kidney glucocorticoid-receptor complexes occurs in vivo. Arch. Biochem. Biophys. 202, 374 - 379.

Maul, G.G., Maul, H.M., Scogna, J.E., Lieberman, M.W., Stein, G.S., Hsu, B.Y., Borun, T.W., (1972) Time sequence of nuclear pore formation in phytohemagglutinin- stimulated lymphocytes and in HeLa cells during the cell cycle. J. Cell Biol. 55, 433 - 447.

Maundrell, K., Maxwell, E.S., Puvion, E. and Scherrer, K., (1981) The nuclear matrix of duck erythroblasts is associated with globin mRNA coding sequences but not with the major proteins of 40S nuclear RNP. Exp. Cell Res. 136, 435 - 445.

McBlain, W.A., Toft, D.O. and Shaymala, G., (1981) Transformation of mammary cytoplasmic glucocorticoid receptor under cell-free conditions. Biochemistry 20, 6790 - 6798.

McPartland, R.P., Milholland, R.J. and Rosen, F., (1977) Nuclear binding of steroid-receptor complex to lymphosarcoma P1798 resistant and sensitive cells and effect of Concanavalin A on receptor levels. Cancer Research 37, 4256 - 4260.

Milgrom, E. and Atger, M., (1975) Receptor translocation inhibitor and apparent saturability of the nuclear acceptor. J. Steroid. Biochem. 6, 487 - 492.

Milgrom, E., Atger, M. and Bailly, A., (1976) Interaction of rat-liver glucocorticoid receptor with DNA. Eur. J. Biochem. 70, 1 - 6.

Mizumo, N.S., Stoops, C.E. and Sinha, A.A., (1971a) DNA synthesis associated with the inner membrane of the nuclear envelope. Nat. New Biol. 229, 22 - 24.

Mizumo, N.S., Stoops, C.E. and Peiffer, R.L.Jr., (1971b). Nature of the DNA associated with nuclear envelope of regenerating liver. J. Mol. Biol. 59, 517 - 525.

Monneron, A. and Segretain, D., (1974) Extensive binding of Concanavalin A to the nuclear membrane. Febs. Lett. 42, 209 - 213.

Morrissey, J.H., (1981) Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem. 117, 307 - 310.

Moudgil, V.K. and Toft, D.O., (1976) ATP - PPi exchange activity of progesterone receptor. Proc. Nat. Acad. Sci. U.S.A. 73, 3443 - 3447.

Moudgil, V.K. and John, J.K., (1980a) ATP-dependent activation of glucocorticoid receptor from rat liver cytosol. Biochem. J. 190, 799 - 808.

Moudgil, V.K. and John, J.K., (1980b) Interaction of rat liver glucocorticoid receptor with adenosine 5'-triphosphate. Biochem. J. 190, 809 - 818.

Müller, R.E., Traish, A.M. and Wotiz, H.H., (1977) Interaction of receptor estrogen complex (R.E.) with uterine nuclei. J. Biol. Chem. 252, 8206 - 8211.

Müller, R.E., Traish, A. and Wotiz, H.H., (1980) Effects of pyridoxyl 5'-phosphate on uterine estrogen receptor. I. Inhibition of nuclear binding in cell-free system and intact uterus. J. Biol. Chem. 255, 4062 - 4067.

Mulvihill, E.R., Le Pennec, J. and Chambon, P., (1982) Chicken oviduct progesterone receptor: Location of specific regions of high-affinity binding in cloned DNA fragments of hormone-responsive genes. Cell 24,621-632.

Munck. A. and Brinck-Johnson, T., (1968) Specific and nonspecific physicochemical interactions of glucocorticoids and related steroids with rat thymus cells in vitro. J. Biol. Chem. 243, 5556 - 5565.

Munck, A., Wira, C., Young, D.A., Mosher, K.M., Hallahan, C. and Bell, P.A., (1972) Glucocorticoid receptor complexes and the earliest steps in the action of glucocorticoids on thymus cells. J. Steroid Biochem. 3, 567 - 578.

Munck, A. and Foley, R., (1976) Kinetics of glucocorticoid-receptor complexes in rat thymus cells. J. Steroid Biochem. 7, 1117 - 1122.

Munck, A. and Foley, R., (1980) Activated and non-activated glucocorticoid receptor complexes in rat thymus cells: kinetics of formation and relation to steroid structure. J. Steroid Biochem. 12, 225 - 230.

Murakami, N., Quattrociocchi, T.M., Healy, S.P. and Moudgil, V.R., (1982) Effects of sodium tungstate on the nuclear uptake of glucocorticoid-receptor complex from rat liver. Arch. Biochem. Biophys. 214, 326 - 334.

Nenci, I., Fabris, G., Marchetti, E. and Marzola, A., (1980) Intracellular flow of particulate steroid-receptor complexes in steroid target cells. Virchows Arch. B. Cell. Path. 32, 139 - 145.

Nielsen, C.J., Sands, J.J. and Pratt, W.B., (1977) Evidence that dephosphorylation inactivates glucocorticoid receptors. Proc. Nat. Acad. Sci. U.S.A. 74, 1398 - 1402.

Noma, K., Nakao, K., Sato, B., Nishizawa, Y., Matsumoto, K. and Yamamura, Y., (1980) Effect of molybdate on activation and stabilization of steroid receptors. Endocrinology 107, 1205 - 1211.

O'Brien, J.M. and Cidlowski, J.A., (1981) Interaction of pyridoxyl phosphate with glucocorticoid receptors from HeLa S_3 cells. J. Steroid Biochem. 14, 9 - 18.

Okada, T.A. and Comings, D.E., (1980) A search for protein cores in chromosomes: is the scaffold an artifact? Am. J. Hum. Genet. 32, 814 - 832.

O'Malley, B.W., Towle, H.C. and Schwartz, R.J., (1977) Regulation of gene expression in eukaryotes. Ann. Rev. Genet. 11, 239 - 275.

Panyim, S. and Chalkley, R., (1971) The molecular weight of vertebrate histones exploiting a modified sodium dodecyl sulphate electrophoretic method. J. Biol. Chem. 246, 7557 - 7560.

Papamichael, M., Tsokos, G., Tsawdaroglou, N. and Sekeris, C.E., (1980) Immunocytochemical demonstration of glucocorticoid receptors in different cell types and their translocation from the cytoplasm to the cell nucleus in the presence of dexamethasone. Exp. Cell Res. 125, 490 - 494.

Papamichael, M.,Ioannidis, C., Tsawdaroglou, N. and Sekeris, C.E., (1981) Translocation of glucocorticoid receptor from cytoplasm into the nucleus of phytohemagglutinin-stimulated human lymphocytes in the absence of the hormone. Exp. Cell Res. 133, 461 - 465.

Paul, J. and Gilmour, R.S., (1968) Organ specific restriction of transcription in mammalian chromatin. J. Mol. Biol. 34, 305 - 316.

Pavlakis, G.N. and Hamer, D.H., (1983) Regulation of a metallothionein-growth hormone hybrid gene in bovine papilloma virus. Proc. Nat. Acad. Sci. U.S.A. 80, 397 - 401

Payvar, F., Wrange, O., Carlstedt-Duke, J., Okret, S., Gustaffson, J., and Yamamoto, K.R., (1981) Purified glucocorticoid receptors bind selectively in vitro to a cloned DNA fragment whose transcription is regulated by glucocorticoids in vivo. Proc. Nat. Acad. Sci. U.S.A. 78, 6628 - 6632.

Peters, K.E. and Comings, D.E., (1980) Two-dimensional gel electrophoresis of rat liver nuclear washes, nuclear matrix and hnRNA proteins. J. Cell Biol. 86, 135 - 155.

Peterson, S.P. and Berns, M.W., (1978) Chromatin influence on the function and formation of the nuclear envelope shown by laser induced psoralen photoreaction. J. Cell Sci. 32, 197 - 213.

Pfahl, M., Sandros, T. and Bourgeois, S., (1978) Interaction of glucocorticoid receptors from lymphoid cell lines with their nuclear acceptor sites. Mol. Cell. Endocrin. 10, 175 - 191.

Quick, P., (1980) Junctions of polytene chromosomes and the inner nuclear membrane. Experientia 36, 456 - 457.

Reeves, R., Chang, D. and Chung, S., (1981) Carbohydrate modifications to high mobility group proteins. Proc. Nat. Acad. Sci. 78, 6704 - 6708.

Richardson, J.C.W. and Agutter, P.S., (1980) The relationship between the nuclear membranes and the endoplasmic reticulum in interphase cells. Biochem. Soc. Trans. 8, 459 - 465.

Richardson, J.C.W. and Maddy, A.H.,(1980a) The polypeptides of rat liver nuclear envelope. I. Examination of nuclear pore complex polypeptides by solid-state peroxidase labelling. J. Cell. Science. 43, 253 - 267.

Richardson, J.C.W. and Maddy, A.H., (1980b) The polypeptides of the rat liver nuclear envelope. II. Comparison of rat liver nuclear membrane polypeptides with those of the rough endoplasmic reticulum. J. Cell. Science 43, 269 - 277.

Riley, D.E. and Keller, J.M., (1978) The ultrastructure of non-membranous nuclear ghosts. J. Cell Science 32, 249 - 268.

Rizzo, W.B. and Bustin, J., (1977) Lectins as probes of chromatin structure - binding of Concanavalin A to purified rat liver chromatin. J. Biol. Chem. 252, 7062 - 7076.

Robinson, S.I., Nelkin, B.D. and Vogelstein, B., (1982) The ovalbumin gene is associated with the nuclear matrix of chicken oviduct cells. Cell 28, 99 - 106.

Rousseau, G.G., Baxter, J.D., Higgins, S.J. and Tomkins, G.M., (1973) Steroid induced nuclear binding of glucocorticoid receptors in intact hepatoma cells. J. Mol. Biol. 79, 539 - 554.

Rousseau, G.G., Higgins, S.J., Baxter, J.D., Gelfand, D. and Tomkins, G.M., (1975) Binding of glucocorticoid receptor to DNA. J. Biol. Chem. 250, 6015 - 1021.

Sando, J.J., La Forest, A.C. and Pratt, W.B., (1979a) ATP-dependent activation of L cell glucocorticoid receptors to the steroid binding form. J. Biol. Chem. 254, 4772 - 4778.

Sando, J.J., Hammond, N.D., Stratford, C.A. and Pratt, W.B., (1979b) Activation of thymocyte glucocorticoid receptors to the steroid binding form: the roles of reducing agents, ATP, and heat-stable factors. J. Biol. Chem. 254, 4779 - 4789.

Schaltman, K. and Pongs, O., (1982) Identification and characterization of the ecdysterone receptor in Drosophila melanogaster by photoaffinity labelling. Proc. Nat- Acad. Sci. U.S.A. 79, 6 - 10.

Scheer, U., (1972) The ultrastructure of the nuclear envelope of amphibian oocytes. IV. On the chemical nature of the nuclear pore complex material. Zellforsch. Mikrosk . Anat. 127, 127 - 128.

Scheer, U., Kartenbeck, J., Trendelenburg, M.F., Stadler, J. and Franke, W.W., (1976) Experimental disintegration of the nuclear envelope. Evidence for pore-connecting fibrils. J. Cell. Biol. 69, 1 - 18.

Schrader, W.T., Socker, S.H. and Buller, R.E., (1977) in Methods in Enz., Vol 36, pp. 292 - 313, (Eds. O'Malley, B. and Hardman, J.), Academic Press, N.Y.

Segrest, J.P. and Jackson, R.L., (1972) in Methods in Enzymology, Vol. 28, pp. 54 - 63, (Ed. Ginsberg, V.), Academic Press, N.Y.

Sekula, B.C., Schmidt, T.J. and Litwack, G., (1981) Redefinition of modulator as an inhibitor of glucocorticoid receptor activation. J. Steroid Biochem. 14, 161 - 166.

Sekula, B.C., Schmidt, T.J., Oxenham, E.A., DiSorbo, D.M. and Litwack, G., (1982) Dual effects of pyridoxal 5'-phosphate on glucocorticoid-receptor complexes. Biochemistry 21, 2915 - 2955.

Seleznev, Y.M., Volkova, N.G., Medvedeva, L.A., Danilov, S.M. and Smirnov, V.N., (1979) Uptake and distribution of glucocorticoids in cell nuclei of rat heart in cell-free cyctems: Dependence on concentrations and forms of added hormones. J. Mol. Cell. Cardiology 11, 289 - 302.

Sevaljevic, L., Petrovic, S.L. and Petrovic, M., (1979) Binding of Lens culinaris lectin to sea urchin embryo chromatin. Experientia 35, 193 - 194.

Sevaljevic, L., Poznanovic, G., Petrovic, M. and Krtolica, K., (1981) Protein kinase activity and lectin binding ability of the nuclear matrix. Biochem. Int. 2, 77 - 84.

Shaw, L.M.J. and Huang, R.C.C., (1970) A description of two procedures which avoid the use of extreme pH conditions for the resolution of components isolated from chromatin prepared from cerebellar and pituitary nuclei. Biochemistry 9, 4530 - 4541.

Shelton, K.R. and Cochran, D.L., (1978) In vitro oxidation of intrinsic sulfhydryl groups yields polymers of the two predominant polypeptides of the nuclear envelope. Biochemistry 17, 1212 - 1216.

Shelton, K.R., Higgins, L.L., Cochran, D.L., Ruffolo, J.J. and Egle, P.M., (1980a) Nuclear lamins of erythrocyte and liver. J. Biol. Chem. 255, 10978 - 10983.

Shelton, K.R., Guthrie, V.H. and Cochran, D.L.,(1980b) On the variation of the major nuclear envelope (lamina) polypeptides. Biochem. Biophys. Res. Comm. 93, 867 - 872.

Shelton, K.R., Guthrie, V.H. and Cochran, D.L., (1982) Oligomeric structure of the major nuclear envelope protein Lamin B. J. Biol. Chem. 257, 4328 - 4332.

Simons, S.S., Martinez, H.M., Garcea, R.L., Baxter, J.D. and Tomkins, G.M., (1976) Interaction of glucocorticoid receptor-steroid complexes with acceptor sites. J. Biol. Chem. 251, 334 - 343.

Sinha, A.A., Blackard, C.E., Doe, R.P. and Seal, U.S., (1973) In vitro localization of ^{3}H estradiol in human prostatic carcinoma. Cancer 31, 682 - 688.

Sinha, A.A. and Mizuno, N.S., (1977) DNA synthesis associated with a DNA-nuclear-membrane complex from rat liver. Cell. Tiss. Res. 183, 191 - 201.

Sluyser, M., (1969) Interaction of steroid hormones with histones in vitro. Biochim. Biophys. Acta 182, 235 - 244.

Spelsberg, T.C., Webster, R., Pikler, G., Thrall, C. and Wells, D., (1976) Role of nuclear proteins as high affinity sites ("acceptors") for progesterone in the avian oviduct. J. Steroid. Biochem. 7, 1091 - 1101.

Spelsberg, T.C., (1982) in "Biochemical Actions of Hormones", IX, 141 - 204. Academic Press, New York.

Stein, G.S., Roberts, R.M., Davis, J.L., Head, W.J., Stein, J.L., Thrall, C.L., van Veen, J. and Welch, D.W., (1975) Are glycoproteins and glycosaminoglycans components of the eukaryotic genome? Nature 258, 639 - 641.

Stick, R. and Krohne, G., (1982) Immunological localization of the major architectural protein associated with the nuclear envelope of Xenopus laevis oocyte. Exp. Cell. Res. 138, 319 - 330.

Tata, J.R., Hamilton, M.J. and Cole, R.D., (1972) Membrane phospholipids associated with nuclei and chromatin: melting profile, template activity and stability of chromatin. J. Mol. Biol. 67, 231 - 246.

Tata, J.R., (1982) Do steroid receptors recognize DNA sequences? Nature 298, 707 - 708

Teng, C.S., Teng, C.T. and Allfrey, V.G., (1971) Studies of nuclear acidic proteins. J. Biol. Chem. 246, 3597 - 3609.

Thrall, C.L., Webster, R.A. and Spelsberg, T.C., (1978) in "The Cell Nucleus", Vol.6, pp. 461 - 529, (Ed. Busch, H.), Academic Press, N.Y.

Thrall, C.L. and Spelsberg, T.C., (1980) Factors affecting the binding of chick oviduct progesterone receptor to deoxyribonucleic acid: evidence that deoxyribonucleic acid alone is not the nuclear acceptor site. Biochemistry 19, 4139 - 4148.

Traish, A.M., Müller, R.F. and Wotiz, H.H., (1977) Binding of estrogen receptor to uterine nuclei. J. Biol. Chem. 252, 6823 - 6830.

Tsai, C. and Frasch, C.E., (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. 119, 115 - 119.

Tsawdaroglou, N.G., Govindan, M.V., Schmid, W. and Sekeris, C.E., (1981) Dexamethasone-binding proteins in cytosol and nucleus of rat thymocytes. Purification of three receptors. Eur. J. Biochem. 114, 305 - 313.

Unwin, P.N.T. and Milligan, R.A., (1982) A large particle associated with the perimeter of the nuclear pore complex. J. Cell Biol. 93, 63 - 75.

van Eekelen, C.A.G. and van Venrooij, W.J., (1981) hnRNA and its attachment to a nuclear protein matrix. J. Cell Biol. 88, 554 - 563.

Vigneri, R., Goldfine, I., Wong, K., Smith, G.J. and Pezzino, V., (1978) The nuclear envelope. The major site of insulin binding in rat liver nuclei. J. Biol. Chem. 253, 2098 - 2103.

Virtanen, I. and Wartiovaara, J., (1976) Lectin receptor sites on rat liver cell nuclear membranes. J. Cell Science 22, 335 - 344.

Virtanen, I., Brotherus, J., Renkonen, O. and Wartiovaara, J., (1977) Phospholipids of outer and inner nuclear membranes in rat liver and BHK - 21 cells. Biochem. Biophys. Res. Comm. 76, 142 - 149.

Virtanen, I., (1977) Identification of Concanvalin A binding glycoproteins of rat liver cell nuclear membranes. Biochem. Biophys. Res. Comm. 78, 1411 - 1417.

Weilach, D.F.H. and Winzler, R.J., (1974) in Evolving Strategies and tactics in membrane research, pp. 66 - 82. Springer Verlag, Berlin.

Wanatabe, H., Orth, D.N. and Toft, D.O., (1974) Glucocorticoid receptors in mouse pituitary tumour cells. Biochemistry 13, 332 - 337.

Wardi, A.H. and Michos, G.A., (1972) Alcian Blue staining of glycoproteins in acrylamide disc electrophoresis. Anal. Biochem. 49, 607 - 609.

Webster, R.A., Pikler, G.M. and Spelsberg, T.C., (1976) Nuclear binding of progesterone in hen oviduct. Role of acidic chromatin proteins in high affinity binding. Biochem. J. 156, 409 - 418.

Westphal, H.M. and Beato, M., (1980) The activated glucocorticoid receptor of rat liver. Purification and physical characterization. Eur. J. Biochem. 106, 395 - 403.

Westphal, H.M. and Beato, M., (1981) Influence of pyridoxyl 5'-phosphate on the DNA binding activity of steroid hormone receptor and other DNA binding proteins. Febs. Lett. 124, 189 - 192.

Westphal, H.M., Fleischmann, G. and Beato, M., (1981) Photoaffinity labelling of steroid binding proteins with unmodified ligands. Eur. J. Biochem. 119, 101 - 106.

Winston, S. and Sueoka, N., (1980) DNA-membrane association is necessary for initiation of chromosomal and plasmid replication in Bacillus subtilis. Proc. Nat. Acad. Sci. U.S.A. 77, 2834 - 2838.

Wong, K.C., Kornel, L., Bezkorovainy, A. and Murphy, B.E.P., (1973) Isolation of cytoplasmic glucocorticoid-binding proteins from rat liver by means of affinity chromatography, and its partial characterization. Biochim. Biophys. Acta 328, 133 - 143.

Wrange, O., (1976) Characteristics of the corticosterone-receptor complex in rat liver cytosol. Biochim. Biophys. Acta 434, 483 - 489.

Wrange, O. and Gustaffson, J.A., (1978) Separation of hormone-binding and DNA-binding sites of hepatic glucocorticoid receptor by means of proteolysis. J. Biol. Chem. 253, 856 - 865.

Wrange, O., Carlstedt-Duke, J. and Gustaffson, J.A., (1979) Purification of the glucocorticoid receptor from rat liver cytosol. J. Biol. Chem. 254, 9284 - 9290.

Wunderlich, F. and Herlan, G., (1977) A reversibly contractile nuclear matrix. Its isolation, structure and composition. J. Cell. Biol. 73, 271 - 278.

Wunderlich, F., Giese, G. and Bucherer, C., (1978) Expansion and apparent fluidity decrease of nuclear membranes induced by low Ca/Mg. J. Cell Biol. 7, 479 - 490.

Yanker, B.A. and Shooter, E.M., (1979) Nerve growth factor in the nucleus: interaction with receptors on the nuclear membrane. Proc. Nat. Acad. Sci. U.S.A. 76, 1269 - 1273.

Yasuzumi, G. and Tsubo, I., (1966) The fine structure of nuclei as revealed by electron microscopy. Exp. Cell Res. 43, 281 - 292.

Zahler, P.H. and Wallach, D.F.H. (1967) Isolation of lipid free plasma membrane proteins by gel filtration on Sephadex LH 20 using 2-chloroethanol-water as solvent. Biochim. Biophys. Acta 135, 371.

Zahler, P.H. and Weibel, E.R., (1970) Reconstitution of membranes by recombining proteins and lipids derived from erythrocyte stroma. Biochim. Biophys. Acta 219, 320 - 328.

Zbarsky, I.B., (1978) An enzyme profile of the nuclear envelope. Int. Rev. Cyt. 54, 295 - 360.